Vol. XXXIX March, 1950 No. 3

SCIENTIFIC EDITION

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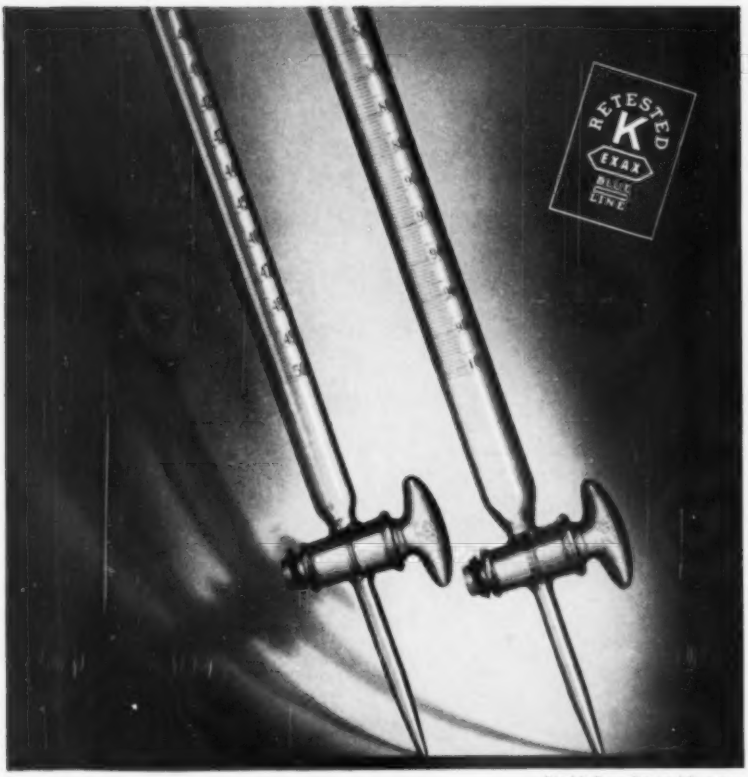

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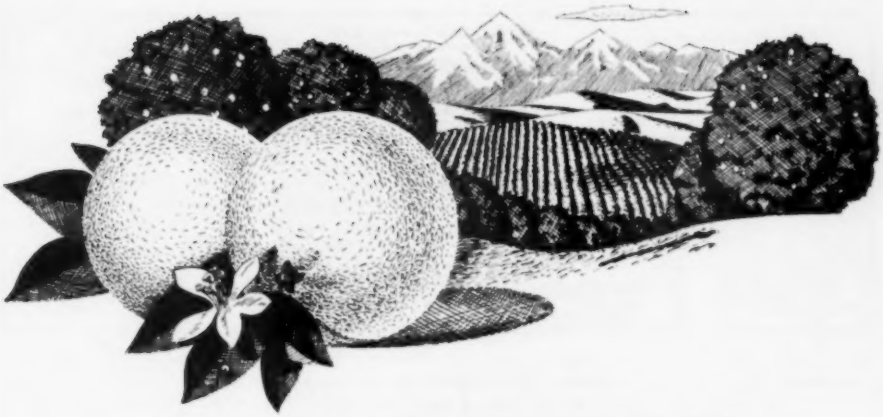

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Published by the American Pharmaceutical Association under supervision of the Committee on Publications with the approval of the Council. Publication office, 20th and Northampton Sts., Easton, Pa. Editorial office: Scientific Edition, 2215 Constitution Ave., Washington 7, D. C.

Annual Subscription—Journal of the American Pharmaceutical Association, complete (both editions): United States and Pan America, $7.00; Canada, $7.70; other foreign, $8.00; members of the American Pharmaceutical Association with dues, $4.00. Each edition, Scientific Edition or Practical Pharmacy Edition: United States and Pan America, $4.00; Canada $4.35; other foreign, $4.50. Single numbers, either edition: United States and Pan America, $0.35; Canada, $0.40; other foreign, $0.50.

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Entered as second-class matter January 23, 1917, at the Post Office at Easton, Pa., under the act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the fifth; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

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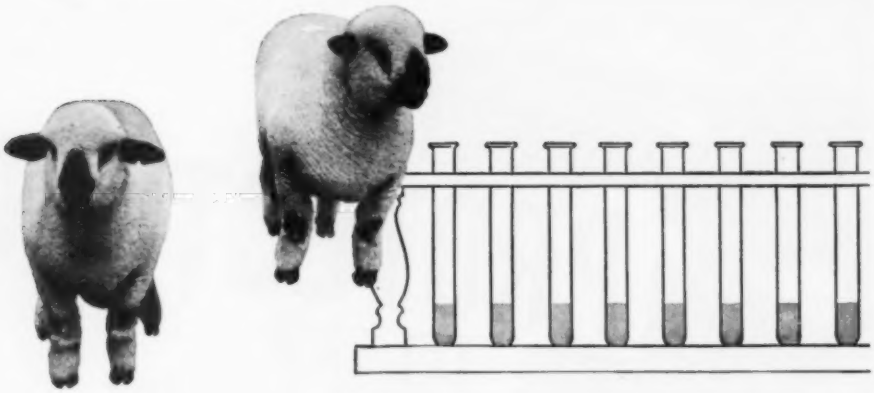

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VOLUME XXXIX — *MARCH, 1950* — NUMBER 3 — CONSECUTIVE NO. 6

Vitamin K_5 as an Antimicrobial Medicament and Preservative*,†

By ROBERTSON PRATT, JEAN DUFRENOY, PETER P. T. SAH, JOHN ONETO, DONALD C. BRODIE, SIDNEY RIEGLEMAN, and VIRGINIA L. PICKERING

Vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride) is a strong antimicrobial agent: a number of pathogenic bacteria and fungi, as well as saprophytic bacterial, yeast, and fungal despoilers of foods, beverages, and other products are markedly inhibited or killed by concentrations of K_5 ranging from 10 to 300 parts per million, depending on the organism. The low toxicity of K_5 suggests its use not only for topical treatment of dermatophytic infections but as a preservative for pharmaceuticals, foods, and beverages. Upon oxidation the compound is subject to discoloration and, in aqueous solution, forms a flocculent precipitate, but this can be obviated by use of appropriate solvents and reducing agents. Vitamin K_5 of high purity can be prepared in almost quantitative yields from vitamin K_3 (2-methyl-1,4-naphthoquinone) which, in turn, is obtained by direct oxidation of 2-methyl-naphthalene.

THE TERM vitamin K, derived from the German expression "Koagulations-Vitamin," has been applied to the compound recognized by Dam (1) in 1929 as specifically decreasing blood clotting time and identified independently by Dam (2) and Almquist and Stockstadt (3) in 1935 as 2-methyl 3-phytyl-1,4-naphthoquinone. With recognition of the fact that other related compounds possess similar antihemorrhagic properties, this compound was designated vitamin K_1.

Vitamin K occurs in leafy vegetables and other natural sources. Several strains of bacteria, notably of the *Bacillus subtilis* and *B. brevis* groups, also biosynthesize naphthoquinones endowed with vitamin K activity; certain strains of *Mycobacterium tuberculosis* produce an antihemorrhagic pigment, phthiocol, related structurally to vitamin K. The members of the K series have been identified from their degradation products as 2-methyl-naphthoquinones with various groups substituted on carbon 3 and/or elsewhere in the molecule.

Several analogs of vitamin K have been synthesized and one of these, 2-methyl-4-amino-1-naphthol hydrochloride (vitamin K_5), has been found to be not only a superior antihemorrhagic agent for clinical use, but also to exhibit marked inhibitory activity toward a number of parasitic and saprophytic microorganisms. The antimicrobial activities of vitamin K_5 have been under investigation in our laboratories for more

* Received July 2, 1949, from the University of California College of Pharmacy, San Francisco.
† This research was supported in part by a generous research grant from the Cutter Laboratories, Berkeley, Calif.

than two years to determine suitability of the compound for use as a chemotherapeutic agent in infections; suitability of the compound as a preservative for foods, beverages, and other economic products; and practical means of applying the compound to these ends. The present paper embodies results of researches which were organized in three divisions as follows:

1. Chemical studies directed toward developing improved methods of synthesis.
2. Biological investigations to ascertain the antimicrobial spectrum of K_5, the concentrations required to inhibit bacteria, yeasts, and molds, and to discover potential practical applications.
3. Pharmaceutical studies to determine suitable bases and vehicles in which to disperse the compound and satisfactory means of applying same.

A number of naphthoquinone derivatives, all possessing a methyl group in position 2, are endowed with antibiotic as well as with antihemorrhagic properties and may be considered as belonging to the vitamin K group.

Vitamin K_5 was considered the most interesting of this group for our studies and was selected for investigation for the following reasons: screening tests showed that it is highly effective in low concentrations as an antimicrobial agent; compared with other members of the series it is relatively soluble in water; and it has a low order of toxicity, the acute LD_{50} for rats and mice being more than 600 mg. K_5, when administered orally and more than 750 mg. K_5 when administered intraperitoneally; after receiving daily oral doses in excess of 350 mg. K_5 for several weeks young rats exhibit no symptoms of toxicity. The solubility characteristics and low toxicity of K_5 recommended the compound for study not only as a drug, but as a preservative for pharmaceuticals, foods, and beverages.

CHEMICAL SYNTHESIS OF VITAMIN K_5

The antihemorrhagic activity of synthetic 1-amino-2-methyl-1-naphthol was mentioned by Doisy, *et al.* (4) in 1939; in 1940 Sah (5) published the first detailed account of a chemical synthesis of vitamin K_5 or 2-methyl-4-amino-1-naphthol hydrochloride utilizing the following five-step procedure: (I) 2-methyl-naphtholactic was nitrated to 1-nitro-2-methyl-naphthalene; (II) the nitro compound was reduced to 2-methyl-1-naphthylamine; (III) the amine was diazotized and decomposed with steam to 2-methyl-1-naphthol; (IV) the naphthol was coupled with diazotized sulfanilic acid to form an azo dye; (V) the azo dye was reduced with stannous chloride and concentrated hydrochloric acid to 2-methyl-4-amino-1-naphthol hydrochloride or vitamin K_5. The next year a simplified method for the synthesis of vitamin K_5 by a four-step procedure (6) was described as follows: (I) α-naphthol was condensed with isatin α-anilide to form a blue dye, 2-naphthalene-2'-indole-indigo; (II) the dye was decomposed by 10% sodium hydroxide solution to sodium anthranilate and 1-hydroxy-2-naphthaldehyde, the latter compound being isolated by steam distillation; (III) the phenolic aldehyde was reduced by the method of Clemmensen (7) to 2-methyl-1-naphthol; (IV) the naphthol was nitrosated to 2-methyl-4-nitroso-1-naphthol and, without isolation, the crude product was reduced with stannous chloride and concentrated hydrochloric acid to vitamin K_5.

A more rapid and less tedious procedure was devised to replace the two earlier methods (8), which are chiefly of theoretical interest. Vitamin K_3 (2-methyl-1,4-naphthoquinone), obtained by the direct oxidation of 2-methyl-naphthalene, was condensed with hydroxylamine to form a monoxime. Without isolation, the product was isomerized with dilute sodium hydroxide to the sodium salt of 2-methyl-4-nitroso-1-naphthol, which was immediately reduced with stannous chloride and concentrated hydrochloric acid to yield K_5 in pinkish white needles which melted without decomposition at 280°. The yield was 30%. Subsequently, a modified method was described in which a monoxime was prepared (80% yield) from 2-methyl-1,4-naphthoquinone and hydroxylamine hydrochloride and the crude monoxime was quantitatively reduced to vitamin K_5 by stannous chloride and concentrated hydrochloric acid (9). The mechanisms of the reactions in the different syntheses and detailed procedures have been reviewed (10).

Since the observations of Pratt, *et al.* (11-13), suggested the possibility of using vitamin K_5 as a food preservative, and since the practicability of such an application would depend, in part, on methods of synthesis capable of giving a high yield of a pure product, Okeio and Sah (14) reinvestigated Sah's original method of preparation and arrived at the following conclusions:

1. Vitamin K_5 may be readily prepared directly from vitamin K_3 via the monoxime without isolation of the intermediate product.
2. The yield of crude vitamin K_5 is almost quantitative.
3. The crude product is efficiently purified by the use of decolorizing charcoal (Norite), sodium bisulfite, hydrochloric acid, and water. From 100 Gm. of vitamin K_3, it is possible to obtain, after decolorization and recrystallization, 80 Gm. of highly purified crystalline vitamin K_5 in the form of white needles, melting with decomposition between 280° and 282°.
4. The isomerization of the monoxime with dilute sodium hydroxide to the sodium salt of 2-methyl-4-nitroso-1-naphthol prior to reduction is not necessary.
5. Washing of the final product with ether is to be avoided since ether readily forms a peroxide in the presence of air and this may catalyze the oxidation of vitamin K_5 to a colored substance.
6. If carefully protected from sunlight, vitamin K_5 is very stable. The slightly pinkish color which sometimes develops on standing does not alter the biological properties of the vitamin.

MICROBIOLOGICAL INVESTIGATIONS

Independently, Pratt, *et al.* [11, 15], Schwartzman [16], and Band [17] in 1948 reported inhibition of microbial activity and growth in media containing low concentrations of K_1. Data averaged from a large number of experiments of the present series are presented in Table I. These show the order of magnitude of K_1 concentrations required to completely inhibit growth of representative pathogenic and saprophytic microorganisms in rich nutrient media.

Vitamin K_1 as a Potential Fungicide for Clinical Use. Following observation that growth of *Trichophyton mentagrophytes* is completely inhibited on Sabouraud's agar medium containing 10 mg vitamin K_1 per liter, this organism and *Microsporum audouini* were selected for further study as representative dermatophytes.

A virulent strain of *Trichophyton mentagrophytes* was isolated in the University of California dermatology clinic from a patient who had received no prior medication. The effect of K_1 on growth of this organism was studied using two different techniques. One consisted of the well known agar cup plate procedure in which a well is made in the center of a "seeded" agar plate by removing a plug of agar with a sterile cork borer and then the solution to be tested is placed in the well. Following a suitable incubation period, the effectiveness of different members of a series of compounds may be compared in terms of the size of the zone in which growth is inhibited around the central well. Averaged results of a number of such tests with K_1 are shown in Table II.

On the basis of these tests vitamin K_1 appeared to be superior to the control preparations as an antifungal agent, at least against *Trichophyton mentagrophytes*. Comparative tests with a number of other antimycotic compounds, to be reported in a later paper, showed K_1 to be as effective as the best ones available and more effective than most.

TABLE I.—CONCENTRATIONS OF VITAMIN K_1 REQUIRED TO PREVENT GROWTH OF DIFFERENT MICROORGANISMS IN NUTRIENT MEDIA[a]

Organism	Concentration	
	Mg. / L.	%
Fungi		
1. *Trichophyton mentagrophytes*	10	0.001
2. *Penicillium notatum*	100	0.010
3. *Aspergillus niger*	100	0.010
4. *Rhizopus nigricans*	250	0.025
Yeasts		
1. *Saccharomyces cerevisiae*	75	0.0075
2. *Saccharomyces ellipsoideus*	100	0.010
Bacteria[b]		
1. *Staphylococcus aureus*	60	0.006
2. *Proteus vulgaris*	80	0.008
3. *Bacillus subtilis*	150	0.015
4. *Pseudomonas fluorescens*	250	0.025
5. *Clostridium botulinum*	200	0.020
6. *Escherichia coli*	100	0.010

[a] Different media were selected for the different organisms according to their nutritive requirements. In each case a medium normally capable of supporting luxuriant growth was used.

[b] The concentrations given for bacteria are killing concentrations, as determined by failure of the organisms to grow upon subsequent plating on rich media. No attempts were made to subculture the fungi or the yeasts.

TABLE II.—ACTION OF VITAMIN K_1 AGAINST *Trichophyton mentagrophytes* IN CUP PLATE TESTS

Preparation	Average diameter of zones of complete inhibition, i.e., no growth[a]
0.1% solution	0.2 to 3 cm., partial inhibition beyond
1.0% solution	6.1 cm., partial inhibition beyond
1.0% ointment	9.0 cm. (full diameter of Petri dish)
Controls	
1% solution zinc undecylenate	2.5-3.0 cm.
Desenex[b]	4.5 cm.

[a] The diameter of the central well in which the different preparations were placed was 1.3 cm.

[b] Desenex, a commercially available "fungicidal preparation for athlete's foot," contains 20% zinc undecylenate and 2% undecylenic acid; the remainder is a microbiologically inert carrier.

Agar cup plate tests are convenient for routinely and rapidly screening a large number of compounds, but they are characterized by certain inherent disadvantages: differences in absorbability of different compounds on the agar and differences in molecular size may lead to unwarranted conclusions in some cases; furthermore, such tests, at best, can be interpreted as indicating stasis. To determine cidal activity it is necessary to plate out subcultures from the area of inhibition.

More illuminating results may be obtained from "skin-scale" tests which more closely simulate clinical tests: scales of skin are removed from infected areas, preferably of patients who have received no prior medication, are cut to approximately uniform size (1 to 2 mm. square), and are immersed for specified times (0.5, 1.0, 2.0, 4.0 hours, etc.) in the solution to be tested. Then the scales are rinsed several times in fresh portions of water. Controls consist of scales immersed in water for a like period of time and similarly rinsed. All scales are transferred to Petri dishes containing nutrient medium and, after a suitable period of incubation, counts are made of the number of scales from which the fungus grows out over the plate. Failure of any growth to occur from a particular fragment in two to three weeks at 25° is taken as evidence that the fungus spores or mycelium contained thereon have been killed by the experimental treatment. Growth is apparent from controls in from four to seven days. The results are expressed as a percentage of the total number of fragments employed from which any growth is evident, that is, the total number of fragments from which growth occurs is divided by the total number of fragments in the particular test and the quotient is multiplied by 100. Several skin scales are tested in each set and the experiments are run in several replicates.

In all tests no growth developed from scales immersed in a 1% aqueous solution of K_1 for one hour, while fungus colonies developed abundantly from all control scales immersed in water. Therefore, it may be concluded that K_1 killed the fungus in these experiments. Identical results were obtained in experiments with 1% solutions of K_1 in human serum.

Experiments with *Microsporum audouini* were conducted in the same way as the skin scale tests, substituting infected hairs from school children who had received no prior medication, for the infected scales. Since salicylanilide (5% solution) is commonly used in cases of ringworm due to *M. audouini*, this compound was selected for comparison with K_3. Averaged results of the experiments (see Table III) show a marked inhibitory effect of 1% solutions of K_3 on *M. audouini* after exposure of the fungus for 0.5 to 2 hours and indicate that after exposure for four hours the spores and mycelia were dead. These results are in sharp contrast to the results obtained with 5% solutions of salicylanilide which, in these experiments, showed no evidence of inhibiting, let alone killing, the spores or mycelia of the mold on freshly isolated hairs.

No skin irritation or other untoward reactions were observed when ointments or solutions containing from 1 to 5% K_3 were applied daily for one week to uninfected volunteer college students.

The above observations seem to warrant clinical testing of vitamin K_3 as a chemotherapeutic agent for treatment of athlete's foot and ringworm caused, respectively, by *Trichophyton mentagrophytes* and *Microsporum audouini*. Such tests now in progress are providing encouraging results.

Vitamin K as an Antimicrobial Agent of Potential Industrial Importance. As was pointed out above, the strong antimicrobial activity of vitamin K_3 and its low toxicity suggest use of this compound as a preservative for certain pharmaceuticals, foods, beverages, and other products of economic importance. Several species of fungi, yeasts, and bacteria were selected as representative common despoilers.

Inhibition of Fungi. In one series of experiments, heavy spore suspensions of fungi were inoculated into media consisting of 4% corn steep solids fortified with mineral salts and lactose and containing in addition different amounts of vitamin K_3. The initial *p*H of all solutions was adjusted to 5.5.

The tests with each species of mold were repeated several times, always with the same result. As would be expected, there was some species variation in sensitivity to K_3; growth of *Penicillium notatum* and of *Aspergillus niger* was completely prevented in solutions containing 100 mg. K_3 per liter for four weeks at which time the experiments were discontinued; a concentration of 250 mg. K_3 per liter completely inhibited growth of *Rhizopus nigricans* (see Table I). A photograph of typical results with *Penicillium notatum* is reproduced in Fig. 1.

Fig. 1.—Growth of *Penicillium notatum* on a culture solution containing corn steep liquor, mineral salts, and different amounts of vitamin K_3. The concentrations of K_3 are indicated in mg. L. at the bottom of each bottle. Photograph taken two weeks after inoculation. Growth was markedly retarded by a concentration of 50 mg./L. and was checked completely by a concentration of 100 mg. L. (From Pratt, *et al.*, *Proc. Natl. Acad. Sci.*, **34**, 523(1948).)

Another series of experiments was designed to determine the usefulness of K_3 as a preservative for dried fruit.

The contents of a package of dried prunes, as obtained from a grocery store, was divided into three groups: one group was soaked for one hour in distilled water, the other two groups were soaked for one hour in an aqueous solution of K_3, one set being immersed in a 0.01% solution, the other in a 0.1% solution. Then all the prunes of all three sets were rinsed in several changes of distilled water, following rinsing, all the prunes were placed in the same moist chamber in an incubator at 28°. In a few days colonies of mold began to appear on the controls soaked only in water, the colonies developed rapidly and after two weeks the prunes were entirely covered with a variety of molds and were completely disintegrated. At this time the fruits treated with K_3 were turgid, were not molded, and showed no evidence of spoilage. A photograph taken at the termination of one experiment after seventeen days is reproduced in Fig. 2.

TABLE III.—COMPARISON OF INHIBITORY EFFECT OF 1% SOLUTIONS OF VITAMIN K_3 AND 5% SOLUTIONS OF SALICYLANILIDE ON *Microsporum audouini* IN INFECTED HAIR TESTS

Time of Exposure	Percentage of Hairs Showing Growth of Fungus After							
	3 Days		5 Days		7 Days		10 Days	
	K_3	Salicylanilide	K_3	Salicylanilide	K_3	Salicylanilide	K_3	Salicylanilide
Controls (immersed in solvent[a] for periods ranging from 0.5 to 4 hr.)	100	100	100	100	100	100	100	100
0.5 hr.	50	100	50	100			100	100
1.0 hr.	0	100	33	100	33		33	100
2.0 hr.	0	100	25	100	25		25	100
4.0 hr.	0		0		0		0	

[a] Solvent consisted of a 70:30 (v:v) mixture of 95% ethanol and acetone.

Tests were made with seeds of peas, hybrid corn, cucumbers, yellow mustard, cotton, and nasturtiums to determine whether K_3 might be used to prevent molding and rotting. Several methods of applying K_3 to the seeds were tried. Best results were obtained by shaking the seeds for five to ten minutes in a flask containing a mixture consisting of 100 mg. K_3 and 9 Gm. Carbowax 6000. Seeds so treated germinated well when placed on moist filter paper in Petri dishes incubated at 26° and they remained free from contamination for ten days, when the experiments were terminated, at that time the control seedlings were, in most cases, completely covered with dense mycelial felts of *Penicillium*, *Aspergillus*, *Rhizopus*, and other common airborne fungus and bacterial contaminants. Prolonged exposure to the K_3-Carbowax mixture was not detrimental to the seeds; after three months of storage in this mixture they germinated better than the controls. It appears, therefore, that vitamin K_3 may be potentially useful in agriculture as a seed disinfectant.

Inhibition of Yeasts. Vitamin K_3 completely blocks growth of several yeasts in various media; for example, *Saccharomyces uviformis* is completely inhibited by 100 mg. K_3 per liter and *S. cerevisiae* by 7.5 mg. per liter in nutrient media.

The effect of different concentrations of vitamin K_3 on production of CO_2 by *S. cerevisiae* in 10% glucose was studied manometrically. The averaged results of several experiments, carried out in solutions adjusted to *p*H 4.8, are shown in Fig. 3. Since the suspensions of yeast cells were placed in vessels with no KOH or other agent to absorb the gas that was liberated, an increase in pressure (curves with a positive slope) may be interpreted to mean that evolution of CO_2 exceeded consumption of O_2 and a decrease in pressure (curves with a negative slope) may be interpreted to indicate the opposite condition, i.e., consumption of O_2 exceeded evolution of CO_2 during the period of measurement.

The data plotted in Fig. 3 show that vitamin K_3 at a concentration of 25 mg. per liter increased absorption of O_2 relative to evolution of CO_2. It is noteworthy that the points describe two straight lines rather than a curve with continuously changing slope. The sharp break in the curve suggests that the availability of a substrate on which rapid O_2 uptake depends was diminished until its concentration fell below a certain threshold, at which time another substrate must have come into play.

A similar but less pronounced excess of O_2 uptake relative to CO_2 evolution was apparent in the presence of 50 mg. K_3 per liter. At a concentration of 100 mg. per liter the relative excess of O_2 uptake became apparent after an initial lag period of approximately one-half hour and continued for approximately two hours. Then the curve of slight negative slope reached the initial zero line where it remained throughout the duration of the experiment, due either to restoration of an RQ of 1 or to com-

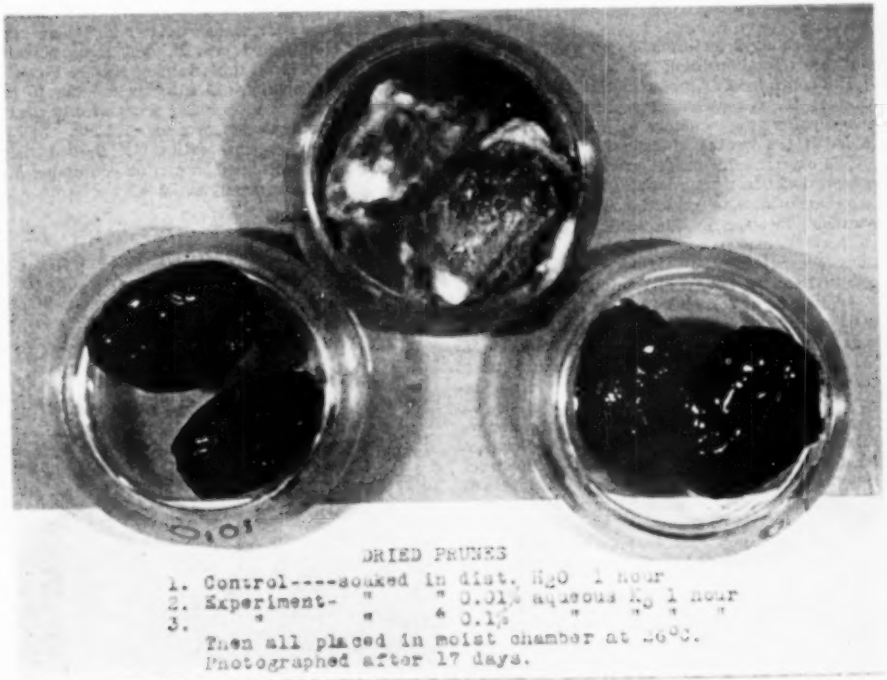
0.01
DRIED PRUNES
1. Control----soaked in dist. H2O 1 hour
2. Experiment- " " 0.01% aqueous K3 1 hour
3. " " " 0.1% " " "
Then all placed in moist chamber at 26°C.
Photographed after 17 days.

Fig. 2.—Note that growth of mold is prolific on the control prunes and on the juice which has exuded from them while the fruit treated with K_3 is turgid and uncontaminated. The bright spots on the treated fruits are reflected high lights. See text for description of experiment.

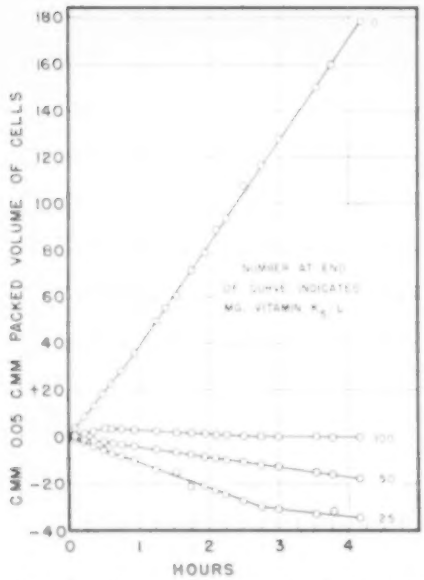

Fig. 3.—Change in gas pressure produced by yeast cultures in 10% glucose with different concentrations of vitamin K_1. [Redrawn from Pratt, *et al.*, *Proc. Natl. Acad. Sci.*, **34**, 325 (1948).]

plete blocking of gas exchanges between the cells and the external medium.

The control curve shows that in the absence of vitamin K_1 a constant RQ greater than 1 was reached after approximately one hour and was maintained for the duration of the experiment.

The experimental results are reminiscent of the observation that appropriate concentrations of naphthoquinones or related compounds induce a transient increase in the rate of utilization of intracellular materials in algae, but hinder the use of substances entering the cell from outside and thereby eventually disrupt metabolic activity (18).

Vitamin K_1 at concentrations as high as 100 mg. per liter did not inhibit the ability of the yeast to reduce colorless triphenyltetrazolium chloride to the red formazan, a reaction which is considered to depend upon dehydrogenase systems requiring coenzymes I and II (19). Conversely, solutions containing 25 mg. or more of K_1 per liter inhibited the indophenol oxidase system in yeast. Normally yeast cells promote rapid formation of indophenol blue from a mixture of dimethyl *p*-phenylene diamine and thymol; however, after having been exposed to solutions containing 25 to 100 mg. K_1 per liter, they not only tend to prevent formation of indophenol blue but, if brought into the presence of the dye, they reduce it.

Development of cloudiness in dry white wines frequently causes serious trouble for producers and distributors; recent research has revealed that there are three principal biological causes of this problem—namely, the yeasts *S. chevalieri*, *S. turbidogenes*, and *S. mellacens*. Therefore, in view of the results recorded above, it seemed advisable to test K_1 as a potential preservative for wines.

The Italian Swiss Colony Wineries kindly supplied us with California Sauternes, titrating 12% alcohol. Samples of wines were dispensed in test tubes. Controls consisted of:

1. Tubes containing wine only.
2. Tubes containing wine inoculated with the yeast under study.
3. Tubes containing inoculated wine fortified to 14% alcohol.

Control number 3 was deemed necessary because stock solutions of K_1 for this experiment were prepared in alcohol and addition of aliquots of this stock solution to the wines raised their alcohol content, the maximum concentration after addition of the K_1 solution being 14%.

Following heavy inoculation with *S. ellipsoideus* the contents of tubes 2 and 3 became turbid after two or three weeks of incubation at 25°.

No macroscopic or microscopic evidence of growth was observed in any of the experimental tubes containing wine with vitamin K_1 added to give concentrations of 10 to 200 mg. per liter. No alteration of color or of taste was apparent.

At first neither *S. chevalieri* nor *S. turbidogenes* grew well in undiluted wine under the conditions of our experiments; but they could be induced to grow in straight wine after a four-day conditioning period in wine diluted with an equal volume of nutrient broth. Following the conditioning, the organisms were inoculated into aliquots of wine with and without K_1 as in the above experiments; after incubation for three weeks at 25° there was abundant sedimented growth, and some pellicle surface growth in all tubes lacking K_1 (see Fig. 4). Wine in the experimental tubes containing 10 or more milligrams of K_1 per liter was perfectly clear. There was no distortion of color or taste. In all experiments subsequent plating out showed that the organisms inoculated into wine containing K_1 were killed and not merely inhibited.

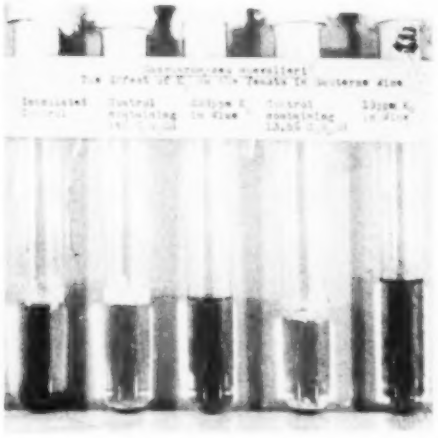

Fig. 4.—Note surface growth and sedimented growth of yeast in control tubes. Wine containing K_1 shows no evidence of contamination. See text for description of experiment.

Similar experiments were carried out using commercial grape juice procured from a grocery store. Different aliquots of the juice were fortified with different amounts of K_3 from an aqueous stock solution; then the juice was dispensed into test tubes, inoculated heavily with *S. chevalieri*, and incubated at 26°. In a few hours fermentation became very active in control tubes containing no K_3, and after twenty-four hours the surface of the liquid was covered with a thick layer of foam. Moderate fermentation was evident after three days in juice containing 10 or 25 mg. K_3 per liter; barely evident fermentation was detectable in tubes with 50 mg. per liter. No evidence of fermentation was found in juice containing 100 mg. K_3 per liter. This concentration of K_3 did not alter the taste or color of the grape juice.

Inhibition of Bacteria.—That the antimicrobial activity of vitamin K_3 is not limited to fungi and yeasts is shown in Table I. Different species of saprophytic and parasitic or facultatively parasitic bacteria that often cause spoilage of foods or beverages are prevented from growing in rich nutrient media when K_3 is added in concentrations ranging from 60 to 500 mg. per liter.

A graded series of concentrations of K_3 was tested against each organism and growth curves were followed by means of a Coleman spectrophotometer. The concentrations given in Table I are end points which completely prohibited growth. That these are cidal and not merely static concentrations was confirmed in each case by subsequently plating the organisms on appropriate media, whereupon no growth occurred.

PHARMACEUTICAL STUDIES

2-Methyl-1-amino-4-naphthol hydrochloride was first synthesized in an attempt to obtain a water-soluble vitamin K. The compound is sufficiently soluble for intravenous use as an antihemorrhagic, since it is effective in doses as low as 1 mg. or less. One per cent aqueous solutions can be prepared (pH approximately 3.2), but the compound rapidly undergoes oxidation and possibly polymerization, resulting in precipitation of a dark brown sediment, unless protected by an antioxidant.

Although complete oxidation of K_3 is undesirable from the pharmaceutical viewpoint, it should be pointed out that complete suppression of oxidation probably inhibits its antimicrobial activity, which is assumed to depend on formation of a quinoid structure. Therefore, an antioxidant which regulates the rate of oxidation without completely blocking the reaction should be selected.

Ointments.—It is well known that water-soluble antibiotic agents such as penicillin are readily released from oil-in-water bases or from hydrogels, but not from water-in-oil emulsions or from water-free bases. In conformity with this general principle, agar plate assays showed that vitamin K_3 is not released from a petrolatum base, but is released from Carbowax or other hydrophilic bases.

When dispersed in a petrolatum base, vitamin K_3 is partially protected from oxidative discoloration, but when incorporated in Carbowax it discolors rapidly unless an antioxidant is added. Sodium metabisulfite, dithiobiuret,[1] propyl gallate,[2] and ascorbic acid were tested as antioxidants for K_3 in various ointment bases as shown in Table IV. The concentration of K_3 was 1% in all ointments.

The most satisfactory ointment, containing 1% K_3, was the Carbowax base II containing 0.4% ascorbic acid; most satisfactory packaging was in collapsible tin tubes, since these afforded minimum contact of the ointment with air.

Testing of such an ointment by the agar cup plate technique, using *S. aureus* as the test organism, showed that activity was undiminished after five weeks at room temperature and one additional week at 104° F., when the experiment was terminated. The ointment melted at the elevated temperature, but upon subsequent cooling and congealing was only slightly discolored. For use in areas characterized by prolonged high temperatures, the consistency of the base should be modified by incorporating Carbowaxes of higher melting points.

Solutions.—Vitamin K_3 may be used conveniently in solution for treatment of certain dermatophytic infections, particularly those of the hair and scalp. Propylene glycol is an excellent solvent for this purpose. Addition of small amounts of anhydrous methyl salicylate to a propylene glycol solution of K_3 tends to prevent discoloration; furthermore, this procedure facilitates penetration of the medicated solution to the site of action.

[1] Kindly provided by the American Cyanamid Company.
[2] Kindly provided by the Heyden Chemical Corporation.

TABLE IV.—COMBINATIONS OF ANTIOXIDANTS AND OINTMENT BASES STUDIED IN VITAMIN K_3 EXPERIMENTS

Sodium metabisulfite	0.1%, in Univ. Calif. base[a] (2)
	0.16%, in Carbowax base I[b]
Dithiobiuret	0.1%, in Univ. Calif. base[a]
	0.2–0.4%, in Carbowax base I[b]
Propyl gallate	0.1%, in Univ. Calif. base[a] with and without 0.05% citric acid
	0.1–0.2%, in Carbowax base II[b] with and without 0.05% citric acid
Ascorbic acid	0.4–1.0%, in Univ. Calif. base[a]
	0.1–0.4%, in Carbowax base I[b]
	0.2–0.4%, in Carbowax base II[b]

a Composition			b Composition			c Composition	
Liquid petrolatum	71.4 Gm.		Carbowax 4000	10 Gm.	Carbowax 4000	20 Gm.
White petrolatum	14.3 Gm.		Carbowax 1540	25 Gm.	Carbowax 1540	30 Gm.
Sodium lauryl sulfate	10% sol.		Propylene glycol	15 cc.	Propylene glycol	35 cc.
Cetyl alcohol	4%	14.2 Gm.			Water	15 cc.
Stearyl alcohol	4%					
Distilled water		20.0 cc.				

It is well known that propylene glycol is of pharmaceutical value as a vehicle not only for drugs administered externally but also for various medicaments administered orally and parenterally. Propylene glycol which itself has some antimicrobial properties has been used alone and in association with other preservatives in the food and fruit industries and should be investigated further as a possible solvent for K_3.

Because of the possible economic application of vitamin K_3 as a preservative, it seemed desirable to investigate means of stabilizing aqueous solutions of the compound against the color change and precipitation that accompany oxidation.

Ascorbic acid was used in concentrations ranging from 0.1% to 1.5%, in solutions of K_3 ranging from 0.1% to 1.0%. No color change or precipitation occurred in 0.1, 0.5, or 1% solutions of K_3 for forty-eight hours (at room temperature) when the concentrations of ascorbic acid were, respectively, 0.1, 0.4, and 1.0%, or higher. However, after seven days at 40° all solutions were discolored and contained a dark purple precipitate; ascorbic acid was abandoned as a stabilizer for aqueous solutions.

In short, of the several antioxidants tested, propylene glycol alone, or aqueous solutions containing 12.5% to 25% propylene glycol, particularly if fortified with 0.15% sodium metabisulfite, were most satisfactory. This mixture protected 1% solutions of K_3 from objectionable discoloration and precipitation for two weeks at 104° F., without diminution of antibacterial activity. When such solutions of vitamin K_3 are autoclaved (15 lb. pressure at 120° C. for fifteen minutes) some discoloration and precipitation occur, but there is no loss of antimicrobial activity.

SUMMARY

Vitamin K_3 (2-methyl-4-amino-1-naphthol hydrochloride) is a water-soluble synthetic analog of the naturally occurring antihemorrhagic vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone).

A study was made of vitamin K_3 as a potentially useful agent for clinical treatment of dermatophytic infections and for protection of foods, beverages, seeds, etc., from spoilage due to the activities of microorganisms.

The following microorganisms were studied: *Trichophyton mentagrophytes, Microsporum audouini, Penicillium notatum, Aspergillus niger, Rhizopus nigricans, Saccharomyces cerevisiae, S. ellipsoideus, S. chevalieri, S. carlsbergensis, Staphylococcus aureus, Proteus vulgaris, Bacillus subtilis, Pseudomonas fluorescens, Clostridium botulinum,* and *Escherichia coli*. Among the fungi, *T. mentagrophytes* (inhibited by 0.001 per cent K_3 in nutrient media) was most sensitive; *Rhizopus nigricans*, requiring 25 times as much K_3 for complete inhibition, was least sensitive. The most sensitive bacterial species was *S. aureus* (killed by 0.0006 per cent K_3 in nutrient broth); *C. botulinum* and *E. coli* were least sensitive, being killed only by concentrations of 0.03 per cent K_3 or higher. Yeast species were completely inhibited in glucose solutions containing 0.0075 to 0.01 per cent K_3, but in wine were killed by 0.001 per cent K_3.

Dried fruits and seeds may be protected from microbial decomposition by proper treatment with dilute preparations of K_3.

Several methods for synthesis of vitamin K_3 are reviewed. A major problem in formulation and storage of compounded preparations of K_3 is providing adequate protection against excessive oxidation. Ascorbic acid is a useful antioxidant in ointments containing K_3; sodium metabisulfite is useful in solutions. Propylene glycol alone or propylene glycol (12.5 per cent) in water is a suitable solvent for K_3.

Preparations containing K_3 do not lose antimicrobial activity upon autoclaving or upon storage for several weeks at temperatures as high as 104° F.

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A Convenient Laboratory Preparation of Chlorothymol, N. F. VIII*

By DANIEL R. SATRIANA, MANUEL M. BAIZER, and KURT S. ELLNER

A procedure is described for preparing 6-chlorothymol (1-methyl-3-hydroxy-4-isopropyl-6-chlorobenzene)[1] in 64.5% yield from thymol.

Two principal methods have been employed for the preparation of chlorothymol: (*a*) the isopropylation of "*p*-chloro-*m*-cresol" (1-methyl-3-hydroxy-6-chlorobenzene) and (*b*) the chlorination of thymol. Numerous variants of (*a*) have appeared in the patent literature (1); in all of them a mixture of chlorothymols is produced, and elaborate fractionation is required in order that a pure product may be obtained.

The literature on method (*b*) is scant. Bocchi (2) and Peratoner (3) chlorinated thymol by means of sulfuryl chloride in the absence of a solvent. Neither author reports yields. Briscoe and Robertson (4) prepared chlorothymol by the action of chlorine gas upon thymol in glacial acetic acid. They obtained a yield of about 50 per cent of material which, even after several recrystallizations from light petroleum ether, did not melt above 58°. Tischtschenko (5) obtained an 80 per cent yield of product by leading chlorine into a suspension of thymol in aqueous sodium carbonate.

The chlorination of thymol, dissolved in carbon tetrachloride[2] by means of sulfuryl chloride, was found to proceed smoothly. The one fractional distillation required during the isolation and purification procedure was satisfactorily accomplished by the use of a 9-inch Vigreux column. The yield of chlorothymol conforming to N. F. VIII purity specifications was 61 to 65 per cent.

EXPERIMENTAL

Sulfur dioxide and hydrogen chloride are evolved during the chlorination. Provisions must be made for absorbing or venting these gases.

Chlorination. In a two-liter three-necked flask equipped with mercury-sealed stirrer, reflux condenser, addition tube, and thermometer, 300 Gm. (2.0 moles) of Thymol, U. S. P. XIII was dissolved in 700 cc. of carbon tetrachloride. Solution took place endothermically and the temperature dropped to 7–10°. With stirring, 185 cc. (308 Gm.) of technical sulfuryl chloride was added in the course of about two hours. The temperature rose to about 31° and the solution became deep yellow. The solution was now heated under reflux (79–82° internal temperature) for eight hours and then allowed to stand at room temperature overnight.

Isolation and Purification. The carbon tetrachloride and excess sulfuryl chloride were removed by distillation, using reduced pressure near the end. The residue was fractionated at 18 mm. through a 9-inch Vigreux column. Fraction (I), b. p. below 140°, weighed 96.5 Gm.; fraction (II), b. p. 140–147°, weighed 254.6 Gm. The undistilled residue consisted of 13.0 Gm. of a dark red oil which was discarded.

Fraction (I) was seeded with pure chlorothymol and allowed to stand overnight at room temperature. The crystals were removed by filtration; the liquor was discarded.[3] The chlorothymol was washed with ice-cold xylene (1 cc. for 3 Gm.), then dried *in vacuo* at room temperature and finally in the oven at 40° for twelve to twenty-four hours. The xylene liquor was added to a similar liquor obtained below.

Fraction (II) was melted. Fifty cubic centimeters of xylene was added, the solution was chilled and stirred rapidly and refrigerated for twelve hours. The crystals were removed by filtration and washed with two 10-cc. portions of ice-cold xylene. The xylene washes were added to the mother liquor, the combined solution concentrated at 70 mm., chilled for twelve hours and filtered. The second crop of crystals was washed with xylene as above. Both crops of chlorothymol were dried by the procedure previously described.

Yield Data. A total of 238.0 Gm. (64.5%) of Chlorothymol, N. F.,[4] was obtained, distributed as follows:[5] 16.6 Gm. from fraction (I), 180.2 Gm. from fraction (II) first crop, and 41.2 Gm. from fraction (II) second crop.

SUMMARY

Thymol, U. S. P., was chlorinated by means of sulfuryl chloride in carbon tetrachloride. The chlorothymol, isolated and purified by the procedure described, conformed to N. F. VIII purity specifications. The yield was 61 to 65 per cent.

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* Received June 17, 1946, from the Research Laboratory of the New York Quinine and Chemical Works, Inc., Brooklyn, N. Y.

[1] This is the numbering system followed by *Chemical Abstracts*. Beilstein lists this same compound as 4-chlorothymol.

[2] The use of this solvent has been mentioned (6), but no modern description.

[3] An additional quantity of chlorothymol can be recovered from this liquor after longer storage.

[4] Tests by N. Y. Q. Control Laboratories.

[5] In a number of individual experiments the distribution of crops varied considerably but the yield totaled 61–65%.

The Preparation and Properties of Equine Pituitary Gonadotropin*

By THOMAS J. HALEY†

A method is described for the alkaline extraction of equine pituitary glands which produced gonadotropic hormone, containing only traces of foreign protein but which was contaminated with posterior pituitary extract. Equine pituitary gonadotropin was found to possess a lower potency than that of serum gonadotropin. When injected into sensitized guinea pigs, equine pituitary gonadotropin did not produce anaphylactic shock.

Since the publication of a method for the extraction of a gonadotropin from the blood serum of pregnant mares by Cole and Hart (5) many investigators (1, 3, 7, 9, 13) have continued work upon the problem of extraction and purification of serum gonadotropin. As a result of this work highly purified preparations are available, but even these can cause allergic manifestations in sensitive individuals. Little has been done in the extraction and purification of the gonadotropic substances of the equine pituitary gland probably because of the unavailability of sufficient quantities of such glands and the time required to process them. With the recent publication of a rapid method for the extraction of gonadotropic substances from fresh pituitary glands (14) and the fact that there were sufficient fresh glands available from the local slaughtering houses, it was decided that an investigation of the preparation and properties of equine pituitary gonadotropins should be undertaken.

EXPERIMENTAL

Extraction and Purification. One kilogram of fresh undissected equine pituitary glands was obtained from a local slaughtering house. These glands were refrigerated immediately after their removal from the skull and were subjected to extraction by a modification of the method of Schwenk, Fleischer, and Tolksdorf (14) within seventy-two hours. After mincing by passage through a meat grinder three times, the glands were mixed with 1 L. of cold distilled water, adjusted to *p*H 8.5 with NaOH solution and stirred for four hours in an ice-salt bath. One liter of cold distilled water and 2 L. of cold chloroform were then added, the *p*H adjusted to 5.5 with glacial acetic acid and the mixture stirred for one more hour. This mixture was then centrifuged for thirty minutes at 2880 r. p. m. to separate the various component parts. The red colored aqueous layer was decanted and saved, and the other layers (spent gland and chloroform) discarded. The 1880 cc. of solution was then poured into a beaker containing 500 cc. of cold acetone to precipitate the active substances (gonadotropins and thyrotropin). The precipitate was removed by suction filtration and the filtrate again mixed with another 500 cc. of cold acetone. This precipitation-filtration process was carried out nine times, giving a final acetone content of 71.4%. It was found that the time involved could be greatly reduced if centrifugation for thirty minutes at 2880 r. p. m. were used in place of filtration. The filter paper containing the active substances was placed in a vacuum desiccator over P_2O_5 for twenty-four hours, then removed, ground, weighed, and placed in screw-capped vials. The individual separations were pooled because the potency of total yield was of more interest than that of each individual fraction. The total yield of a light tan powder was 12.5 Gm. or 1.25%, based upon the amount of gland extracted.

In so far as the purity of the preparation was concerned, several points have yet to be clarified, especially since the material was produced from whole pituitary. However, the method of extraction employed makes it probable that only the gonadotropic and thyrotropic hormones of the anterior lobe and the oxytocic and pressor hormones of the posterior lobe would be present in the aqueous extract. Inasmuch as these latter hormones usually are extracted only in the acid phase (15, 16) and are inactivated by alkali, the *p*H 8.5 used in the preliminary extraction might eliminate them from further consideration. Furthermore, the aqueous acetone mixture probably retained a good portion of the thyrotropic hormone, thus leaving an almost pure gonadotropin. It is also possible that gonadotropically inert but antigenic protein material might be carried through the purification process. Millons, Hopkins-Cole's, and the Xanthoproteic protein reactions were faintly positive, while the Biuret reaction was negative. This would indicate that a small amount of protein material was extracted and was present in the final product.

Pharmacological Evaluation. The dried powder was dissolved in normal saline and assayed for posterior pituitary activity by the U. S. P. guinea pig uterine method (15). Two separate assays showed that the material contained 0.08 U. S. P. Units per milligram.

The capillary vasoconstrictive activity of the dried powder was determined using a modification of the Chambers-Zweifach rat mesoappendix preparation (4, 11). The capillary bed was tested for vasoconstrictive activity by applying 0.1 cc. of 2×10^{-6} epinephrine both before and after the topical application of 0.1 cc. (equivalent to 0.1 mg. of the dried

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powder of the gonadotropin. This method demonstrates the peripheral site of action of vasoconstrictive substances because it is possible to observe closure of the precapillary sphincters and a reduced flow in the capillary bed. Evaluation on three such preparations showed that 0.1 mg. of the dried powder was equivalent to 0.1 cc. of 2×10^{-6} epinephrine in vasoconstrictor activity.

Pressor activity was evaluated using the normal rat preparation of D'Amour and Blood (6), recording the pressor responses with a mercury manometer. The four animals used weighed 225 to 385 Gm. All drug injections were made via the jugular vein in a constant volume of 0.1 cc. followed by 0.25 cc. of physiological saline solution. As two of the pressor assays indicated that the gonadotropin extract had a pressor activity of 0.03 unit per milligram, and because the response to the latter was slightly delayed as compared to posterior pituitary extract, two other rats were given intravenous diphenhydramine (1.2 mg./Kg.) to block any response which might have been due to histamine. This procedure did not result in any increase in the height of response to either pituitary preparation, thus indicating that histamine was not involved in either this assay or the guinea-pig uterus evaluation.

The gonadotropic potency of the dried powder was determined by the Cartland-Nelson method (2). Four groups of ten immature female Wistar strain rats, twenty-six days old, weighing 35–40 Gm. each, were injected subcutaneously once daily with a 0.5 cc. dose. The controls received normal saline and the test animals the following total dose of gonadotropins: 3, 6, and 12 mg. All the animals were sacrificed ninety-six hours after the first dose and their ovaries and uteri removed and weighed. Table I gives the results of this assay and the number of animals represents the total of each group minus the males found at autopsy. The dosage levels used were based upon previous evaluation of a beef pituitary preparation (10) prepared similarly and assayed by the method of Levin and Tyndall (12).

When a comparison is made between Cartland and Nelson's (2) results with equine serum gonadotropin and those herein reported, it is apparent that the pituitary product has a low potency, about 1 Cartland-Nelson Unit per milligram. Ward (17), using the method of Cole and Hart (5), has found that further purification of this pituitary product resulted in a two and one-half fold increase in gonadotropic potency, but this still does not approach that of the serum preparation.

Inasmuch as materials obtained from equine sources are known to produce a variety of allergic responses in sensitized individuals, it was decided that this equine pituitary product should be tested for its sensitizing properties. Ten male guinea pigs, weighing 550 Gm. each, were divided into two equal groups. The control group received 1 cc. of normal saline subcutaneously and the test group 1 cc. of a suspension of the test material (20 mg./cc.) by the same route. Fifteen days later both groups received a 0.5 cc. shocking dose of the suspension intramuscularly. No anaphylactic response was obtained, so eight days later all the animals were given a 1 cc. (1 mg./cc.) dose intravenously via the penis vein. Again no symptoms of anaphylaxis were observed. This would indicate that the material contained little or no foreign proteins or, that if sensitization does occur, it requires more injections and a greater period between the sensitizing and shocking doses.

TABLE I.—EFFECT OF EQUINE GONADOTROPIN ON OVARIAN AND UTERINE WEIGHTS

Group	No. of Animals	Total Dose	Total Ovarian Weight, Mg.	Average Ovarian Weight, Mg.	Total Uterine Weight, Mg.	Average Uterine Weight, Mg.
I (Control)	10	0.9 cc. saline	385	38.5	500	50.0
II	7	3 mg.	1515	220.7	1200	171.4
III	9	6 mg.	1370	156.0	1000	111.1
IV	6	12 mg.	1970	328.3	1450	241.6

SUMMARY

1. Equine pituitary glands were extracted by a simple procedure which produced gonadotropic hormone containing very little foreign protein.

2. Equine pituitary gonadotropin produced by this alkaline extraction process was shown to be contaminated with posterior pituitary extract.

3. Equine pituitary gonadotropin is of relatively low potency when compared to equine serum gonadotropin.

4. Equine pituitary gonadotropin did not produce anaphylactic shock upon injection into sensitized guinea pigs.

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Spasmolytics: Basic Esters of Substituted Malonic Acid*,†

By ARTHUR J. McBAY,‡ GLENN L. JENKINS, and JOHN B. DATA

Certain 2-diethylaminoethyl esters of disubstituted malonic acid that resembled effective antispasmodic esters of analogous disubstituted acetic acids have been synthesized. The preparative methods used are described. Preliminary pharmacological studies indicate that all the compounds show some degree of antispasmodic activity.

Atropine (I) is a naturally occurring spasmolytic. The structure of atropine has served as a pattern in the synthetic development of potent spasmolytics which resemble this plant prototype in molecular architecture. Atropine

is an ester of an aminoalcohol, namely, it is an ester of 2-(hydroxymethyl)phenylacetic acid, tropic acid, and the heterocyclic aminoalcohol, tropine.

Three medicinally important synthetic spasmolytics are Trasentine (II), Syntropan (III), and Pavatrine (IV). All are esters of aminoalcohols.

II

III

IV

Trasentine is a basic ester of diphenylacetic acid. Syntropan is a basic ester of 2-(hydroxymethyl)phenylacetic acid. Pavatrine is a basic ester of 9-fluorenecarboxylic acid. The group,

present in atropine, Trasentine, and Syntropan is also present in Pavatrine, except that in Pavatrine the alpha carbon is a part of the fluorene nucleus.

All four of these medicinally useful spasmolytics have the common group

and thus they may be looked upon as derivatives of acetic acid.

Most of the compounds which are esters of aminoalcohols prepared in the search for effective neurotropic or musculotropic spasmolytics may be classified as derivatives of the structure

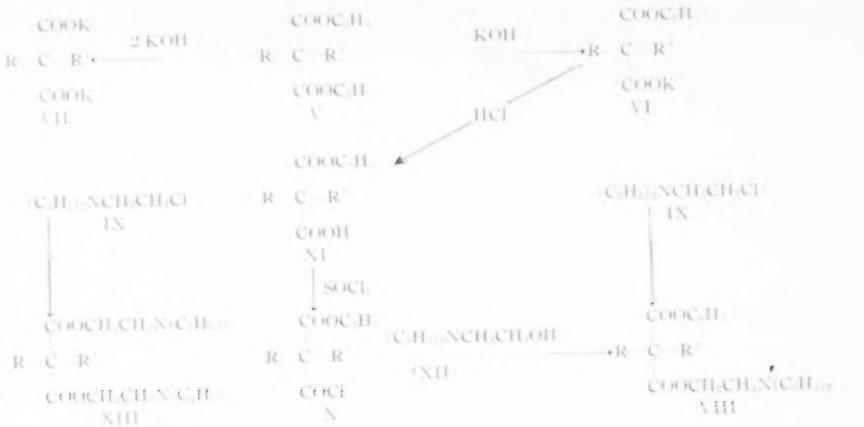
whether the structure be a part of a ring or not, as illustrated in formulas (I), (II), (III), and (IV). Two excellent reviews (1, 2) covering most of this work have been published.

A review of the literature indicates that the physiological properties of basic esters of substituted carboxyacetic acid, malonic acid, have been studied very little. Einhorn, *et al.* (3), prepared bis-2-diethylaminoethyl diethylmalonate but did not report on its physiological properties. Of interest is the report of Gilman and Johnson (4) that bis-2-diethylaminoethyl malonate shows interesting physiological properties. Revelation of these facts prompts us to make a preliminary investigation of the pharmacological properties of a few readily accessible 2-diethylaminoethyl ethyl substituted malonates and bis-2-diethylaminoethyl substituted malonates to ascertain whether these types of compounds are worthy of further investigation.

Nine basic esters of substituted malonic acid have been prepared, and these compounds have been tested for spasmolytic activity by the Magnus method.

The compounds reported here were prepared according to the scheme on page 139.

Diethyl substituted malonates (V), prepared in the usual manner, were allowed to react with one or two equivalents of alcoholic potassium hydroxide to yield the monopotassium salt (VI) and



the dipotassium salt (VII), respectively. The monopotassium salt was converted into 2-diethylaminoethyl ethyl substituted malonate (VIII) through one of two methods: (a) by reaction of the monopotassium salt with 2-diethylaminoethyl chloride (IX), and (b) by the reaction of ethyl substituted malonyl chloride (X), prepared from ethyl hydrogen substituted malonates (XI) and thionyl chloride, with 2-diethylaminoethanol (XII). The dipotassium salt (VII) was allowed to react with 2-diethylaminoethyl chloride (IX) to give bis-2-diethylaminoethyl substituted malonate (XIII).

Preliminary pharmacological studies indicate that all the compounds show some spasmodytic activity against Mecholyl and barium chloride. A detailed report on the pharmacological properties of these compounds is the subject of a forthcoming paper by C. P. Headlee and L. D. Edwards of the Pharmacology Department, School of Pharmacy, Purdue University. The authors wish to acknowledge to these investigators their appreciation for the assistance extended to them in the preliminary pharmacological studies.

EXPERIMENTAL

All melting points are uncorrected. Chlorine was determined by the method described by Blicke and Zienty (5) and nitrogen by a semimicro Kjeldahl method (6).

Preparation of Diethyl Substituted Malonates. The diethyl malonates used in the preparation of certain 2-diethylaminoethyl esters of substituted malonic acid reported in this paper were prepared by methods described in the literature. The procedure described by Adams and Kamm (7) is designated in Table I, "Method I"; and the method described by Cyngell, Phillips, and Smith (8) is designated "Method II." The diethyl-substituted malonates that were prepared are tabulated in Table I.

TABLE I.—DIETHYL SUBSTITUTED MALONATES

R	R′	Method Used	Yield, %	Found: B.P. (°C./Mm.)	Literature: B.P. (°C./Mm.)	Reference
n-Butyl	Hydrogen	I	66	130–135 (20 mm.)	130–135 (20 mm.)	7
Isoamyl	Hydrogen	II	78	126–132 (15 mm.)	102 (3 mm.)	9
Benzyl	Hydrogen	II	61	175–180 (17 mm.)	195 (22 mm.)	10
Isoamyl	Ethyl	II[a]	86	135–139 (15 mm.)	150 (20 mm.)	11
n-Hexyl	*n*-Hexyl	II	82	170–183 (4 mm.)	155–158 (4 mm.)	12
Allyl	Allyl	II[b]	91	135–139 (18 mm.)	207.5–208.5 (200 mm.)	13
Benzyl	Ethyl	II	86	172–174 (10 mm.)	160–170 (9 mm.)	14
Benzyl	*n*-Butyl	I[c]	70	160–165 (15 mm.)	187 (15 mm.)	10
Benzyl	Benzyl	II	76	210–212 (6 mm.)	235–240 (28 mm.)	15

[a] Prepared from diethyl isoamylmalonate.
[b] Diethyl malonate was dialkylated in one step.
[c] Prepared from diethyl benzylmalonate.
[d] Prepared from diethyl *n*-butylmalonate.

Preparation of 2-Diethylaminoethyl Chloride.—To a solution of 117.0 Gm. (1.0 mole) of 2-diethylaminoethanol in 300 ml. of anhydrous benzene, there was added dropwise with stirring during two hours a solution of 155.0 Gm. (1.3 moles) of thionyl chloride in 200 ml. of anhydrous benzene while the temperature of the reaction mixture was maintained at 0° to 5°. The mixture was refluxed for six hours, the solvent and excess thionyl chloride were removed under reduced pressure, and the residue was heated at 100° for two hours under 12–15 mm. pressure. The crystalline mass of 2-diethylaminoethyl chloride hydrochloride, 165–171 Gm., was kept dry until the free base was needed.

The crude hydrochloride was dissolved in 175 ml. of water and the solution was filtered. The filtrate was cooled to 0° in an ice bath, and then there was added to the constantly stirred solution 200 ml. of sodium hydroxide (40%) while the temperature of the reaction mixture was maintained at 0° to 5°. The free base was extracted with 100 ml. of ether, the ethereal solution was dried over anhydrous sodium sulfate, the solvent was removed by distillation, and the residue was fractionated under reduced pressure to give 90.0 to 95.0 Gm. of a colorless liquid with a boiling point of 47–48° (14 mm.). Gough and King (16) reported a boiling point of 51–52° (16 mm.).

Preparation of 2-Diethylaminoethyl Ethyl Dibenzylmalonate Hydrochloride.—To a solution of 85.0 Gm. (0.25 mole) of diethyl dibenzylmalonate in 150 ml. of absolute alcohol, there was added dropwise with stirring a solution of 16.5 Gm. (0.25 mole) of potassium hydroxide (85%) dissolved in 150 ml. of absolute alcohol. After the solution had been stirred for an additional three hours, it was allowed to stand for twenty-four hours at room temperature. The reaction mixture was heated to boiling and filtered, and the filtrate was concentrated to dryness under reduced pressure. The residue was washed with 50 ml. of ether and then dried at 100°. Sixty grams of crude potassium ethyl dibenzylmalonate was obtained.

The salt was dissolved in 100 ml. of water cooled to 5°, and while the solution was stirred, 25 ml. of hydrochloric acid (38%) was added in small portions. During the addition the temperature was maintained below 10°. The oily layer was removed, the aqueous layer was extracted with ether, the ethereal extract was combined with the oil, and the ethereal solution was dried with anhydrous sodium sulfate. Concentration of the ethereal solution yielded 50.0 Gm. of crude ethyl hydrogen dibenzylmalonate.

The acid ester was dissolved in 50 ml. of benzene. The solution was distilled partially until the clearness of the distillate indicated the removal of last traces of water. Thionyl chloride, 20 ml., was added, and the solution was refluxed for five hours. After removal of the low boiling fraction under reduced pressure, the residue was fractionated to give 33.0 Gm. of ethyl dibenzylmalonoyl chloride with a boiling point of 221–225° (10 mm.).

2-Diethylaminoethanol, 12.4 Gm. (0.11 mole), was added to 33.0 Gm. of ethyl dibenzylmalonoyl chloride dissolved in 50 ml. of anhydrous benzene. The reaction mixture was refluxed for five hours and then cooled, 50 ml. of water was added, and the mixture was made alkaline with solid sodium carbonate. The benzene layer was separated, the solvent was removed under reduced pressure, and the residue was fractionated to give 27.0 Gm. of 2-diethylaminoethyl ethyl dibenzylmalonate, boiling range 210–220° (7 mm.).

The base was converted into the hydrochloride salt by passage of dry hydrogen chloride into an anhydrous ethereal solution of the base in the usual way. Recrystallization of the solid from ethyl acetate yielded 10.0 Gm. of pure product with a melting point of 121–122°.

Anal. Calcd. for $C_{25}H_{34}NO_4Cl$: Cl, 7.95; N, 3.13. Found: Cl, 8.05; N, 3.23.

Preparation of 2-Diethylaminoethyl Ethyl Benzyl-α-butylmalonate Hydrochloride.—This product was prepared by the same procedure as described above for the dibenzylmalonate derivative. Diethyl benzyl-α-butylmalonate, 30.6 Gm. (0.1 mole), gave 26.5 Gm. of potassium ethyl benzyl-α-butylmalonate. This salt was converted into 21.0 Gm. of the corresponding acid. The acid gave 18.0 Gm. of the chloride, boiling range 167–173° (8 mm.). The chloride was converted into 15.0 Gm. of the base, boiling range 185–220° (14 mm.). The base was converted into the hydrochloride and then recrystallized from ethyl acetate to give 6.0 Gm. of white, microcrystalline product with a melting point of 96–97°.

Anal. Calcd. for $C_{20}H_{32}NO_4Cl$: Cl, 8.58; N, 3.38. Found: Cl, 8.62; N, 3.33.

Preparation of 2-Diethylaminoethyl Ethyl Benzylethylmalonate Hydrochloride.—A solution of 14.5 Gm. (0.22 mole) of potassium hydroxide (85%) in 80 ml. of absolute alcohol was added dropwise with stirring to a solution of 62.0 Gm. (0.22 mole) of diethyl benzylethylmalonate in 80 ml. of absolute alcohol. The solution was stirred for three hours and then allowed to stand overnight at room temperature. The solution was filtered for the removal of a slight precipitate, 30.0 Gm. (0.22 mole) of 2-diethylaminoethyl chloride was added, and the reaction mixture was refluxed for three hours. The cold solution was filtered for the removal of the precipitated potassium chloride, and the filtrate was fractionated to give 22.0 Gm. of 2-diethylaminoethyl ethyl benzylethylmalonate, boiling range 192–214° (9 mm.).

The base was converted into the hydrochloride salt by the passage of dry hydrogen chloride into an anhydrous ethereal solution of the base in the usual way. Recrystallization of the solid from ethyl acetate yielded 12.0 Gm. of a white, crystalline product with a melting point of 135–136°.

Anal. Calcd. for $C_{20}H_{32}NO_4Cl$: Cl, 9.20; N, 3.63. Found: Cl, 9.25; N, 3.67.

Preparation of 2-Diethylaminoethyl Ethyl Isoamylethylmalonate Hydrochloride.—A solution of 15.5 Gm. (0.24 mole) of potassium hydroxide (85%) in 100 ml. of absolute alcohol was added to 54.5 Gm. (0.20 mole) of diethyl isoamylethylmalonate in 100 ml. of absolute alcohol. The solution was stirred, allowed to stand, and then filtered as in the preparation of 2-diethylaminoethyl ethyl benzylethylmalonate hydrochloride.

2-Diethylaminoethyl chloride, 27.0 Gm. (0.20 mole), was added to the alcoholic solution, and the reaction mixture was refluxed for three hours. The alcohol was removed under reduced pressure, the residue was dissolved in ether, and the ethereal solu-

TABLE II.—BIS-2-DIETHYLAMINOETHYL SUBSTITUTED MALONATE DIHYDROCHLORIDES

$$\begin{array}{c} R \\ | \\ R'-C \\ | \\ COOCH_2CH_2N(C_2H_5)_2 \cdot HCl \end{array}$$

$$\begin{array}{c} R \\ | \\ R'-C \\ | \\ COOCH_2CH_2N(C_2H_5)_2 \cdot HCl \end{array}$$

R	R'	Recryst. Solvent	M. P., °C.	Analyses, %: Chlorine Calcd.	Chlorine Found	Nitrogen Calcd.	Nitrogen Found
Ethyl	Isoamyl	Alcohol-ethyl acetate	155–156	15.10	15.20	5.93	5.93
n-Hexyl	*n*-Hexyl	Alcohol-ethyl acetate	160–161	13.05	13.05	5.17	5.18
Allyl	Allyl	Alcohol-ethyl acetate	157–158	15.55	15.55	6.15	6.05
Benzyl	*n*-Butyl	Alcohol-benzene	157–158	13.60	13.52	5.38	5.53

tion was washed with water and then dried with anhydrous sodium sulfate. The filtered ethereal solution was treated with dry hydrogen chloride, and the precipitate was collected by filtration. The solid was dissolved in hot ethyl acetate and the solution was norited. The filtered solution was cooled, and the resultant precipitate was removed by filtration. The solid was recrystallized several times from ethyl acetate to give 12.0 Gm. of a white, microcrystalline product with a melting point of 119–120°.

Anal. Calcd. for $C_{21}H_{45}NO_4Cl_2$: Cl, 9.72; N, 3.83. Found: Cl, 9.87; N, 3.82.

Preparation of Bis-2-diethylaminoethyl Substituted Malonate Dihydrochlorides. Compounds listed in Table II were prepared by the procedure described below for bis-2-diethylaminoethyl dibenzylmalonate dihydrochloride.

To 85.0 Gm. (0.25 mole) of diethyl dibenzylmalonate, there was added a solution of 50.0 Gm. (0.75 mole) of potassium hydroxide (85%) in 100 ml. of alcohol (50%). The solution was refluxed for twenty hours on a steam bath, and the reaction mixture was then concentrated to dryness under reduced pressure. The residue was washed with ether and then with absolute alcohol. The solid was dried at 100° for twelve hours to give 52.5 Gm. of crude dipotassium dibenzylmalonate.

The dried salt was suspended in 150 ml. of absolute alcohol, and 29.5 Gm. (0.30 mole) of 2-diethylaminoethyl chloride was added. The reaction mixture was refluxed on a steam bath for three hours and then filtered. The alcohol was then removed from the filtrate by distillation under reduced pressure. The crude bis-2-diethylaminoethyl dibenzylmalonate was dissolved in ether, and the ethereal solution was washed with several 50-ml. portions of water and then dried with anhydrous sodium sulfate.

The dihydrochloride was prepared by passage of dry hydrogen chloride into the dried ethereal solution of the base in the usual way. The ether-insoluble dihydrochloride was removed by filtration, the solid was digested with hot ethyl acetate, and the product was recrystallized from alcohol-ethyl acetate to give 15.0 Gm. of a white, microcrystalline solid with a melting point of 152–154°.

Anal. Calcd. for $C_{34}H_{48}N_2O_4Cl_2$: Cl, 12.75; N, 5.05. Found: Cl, 12.71; N, 5.05.

SUMMARY

1. Four 2-diethylaminoethyl ethyl ester hydrochlorides of dibenzylmalonic, benzyl *n*-butylmalonic, benzylethylmalonic, and isoamylethylmalonic acids have been prepared.

2. Five bis-2-diethylaminoethyl ester dihydrochlorides of dibenzylmalonic, benzyl *n*-butylmalonic, isoamylethylmalonic, di-*n*-hexylmalonic, and diallylmalonic acids have been prepared.

3. Preliminary pharmacological studies indicate that all the compounds show some spasmolytic activity.

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The Antispasmodic Action of *Euphorbia pilulifera**

By REBECCA C. HELLERMAN and LLOYD W. HAZLETON

The results of studies reported substantiate a previous postulate that the antispasmodic action of euphorbia is myotropic rather than neurotropic. Since the antispasmodic properties are demonstrated against contractions by cholinergic and histaminic drugs, allergic reactions, and direct muscular action on smooth muscle (ileum, uterus, trachea), they are assumed to be a result of direct muscular action which is completely reversible. It has little, if any, relaxing properties on normal muscle.

For many years *Euphorbia pilulifera* was used as an antiasthmatic (1–5) and was official in the National Formulary until the Eighth Edition. In a recent study on *Euphorbia pilulifera* (6) certain antispasmodic properties, including some measure of efficacy against anaphylactic shock, were revealed. The following investigations were directed at further evaluation of these antispasmodic properties, taking into consideration the role of histamine in anaphylaxis and the pharmacological differences between smooth muscle and the bronchial musculature.

The role of histamine in anaphylaxis has been extensively explored. Dale (7) in his review of the literature expands his original theory to say that there is in all probability both extrinsic and intrinsic histamine in an anaphylactic reaction. Landau, *et al.* (8), and others have shown experimentally that the antihistamines are effective against histamine and to a lesser extent against anaphylactic shock. Gay, *et al.* (9), have shown clinically that these drugs are of value in treating the acute attacks of allergic rhinitis and urticaria but have proved ineffective in the treatment of the more chronic type reaction such as occurs in bronchial asthma. On the other hand, it has long been recognized that antispasmodics such as epinephrine, aminophylline, and papaverine are effective bronchodilators.

Castillo and deBeer (10) have shown that, while bronchodilator drugs such as aminophylline are ineffective on the ileum as previously reported (11, 12), they will relax tracheal chains, both untreated and contracted by histamine. Conversely, they point out that Syntropan and Trasentin, which have been shown to antagonize the histamine contraction of the ileum (11, 13, 14), are ineffective against histamine contractions of the trachea. Castillo (15) has shown that, while the antihistamines are relatively effective against histamine contraction of the trachea, they are ineffective against allergic contractions. The anaphylactic spasms, as well as histamine spasms, can be relaxed by such bronchodilator drugs as aminophylline and epinephrine.

EXPERIMENTAL

The usual techniques were used in studying isolated mouse and guinea-pig ileum and guinea-pig uterus. Segments of tissues were placed in a chamber containing Locke's solution which was maintained at 37° and through which oxygen was allowed to bubble. The lever ratio was 2:1, the pen writing double the excursion of the muscle. The quantities of drugs quoted are those added to the 100 cc. bath and do not represent the final dilution in contact with the muscle.

The preparation of euphorbia used in the experiments was a partially purified dealcoholized fluidextract. This was prepared by evaporating a measured amount of fluidextract of euphorbia to a syrupy consistency over a water bath and making up to the original volume with water. This was then filtered through Lloyd reagent.

Mouse Ileum

Acetylcholine. Euphorbia relaxes and prevents acetylcholine contractions of isolated mouse ileum. Figure 1 shows that 0.2 cc. of acetylcholine (0.1 mg. cc.) added to the bath causes a strong contraction which is relaxed by the addition of 1.0 cc. of euphorbia. After washing, 1.0 cc. of euphorbia was added to the bath. This lowers the baseline very slightly but greatly reduces the acetylcholine contraction. Figure 1 is typical of the action of acetylcholine and euphorbia on both mouse and guinea-pig ileum.

Guinea-Pig Ileum

Barium Chloride. The addition of 0.1 cc. (100 mg. cc.) of $BaCl_2$ to the bath containing a segment of isolated guinea-pig ileum causes a strong contraction which does not relax spontaneously. As shown in Fig. 2, 2.0 cc. of euphorbia added to the bath causes an immediate relaxation. However, there follows a gradual rise which is partially relaxed by an additional 2.0 cc. of euphorbia. This is the typical response obtained.

Guinea-Pig Uterus

Histamine. Euphorbia relaxes a strong histamine contraction of guinea pig uterus. The addition of 0.2 cc. of histamine (1.0 mg. cc.) to the bath causes a strong contraction which is relaxed by the addition of 5.0 cc. of euphorbia to the bath. A contraction by histamine is prevented when 5.0 cc. of euphorbia is added to the bath.

* Received April 25, 1949, from Burlton Laboratories, Falls Church, Va.
Presented to the Scientific Section, A. Ph. A., Jacksonville meeting, April, 1949.

Fig. 1.—(a) Relaxation by euphorbia of an acetylcholine contraction of mouse ileum. (b) Diminution by euphorbia of an acetylcholine contraction.

Fig. 2.—Relaxation by euphorbia of a barium chloride contraction of guinea pig ileum.

Tracheal Chain of Guinea Pig

The tracheal chain method of Castillo and deBeer (10) was used to study further the effect of euphorbia as an antispasmodic. Because of the close similarity, anatomical and physiological, between the tracheal and bronchial musculature, the method is particularly well adapted for the study of bronchodilator drugs. The guinea pig is killed by a blow on the head, the trachea removed and cut into 10 to 12 rings which are tied together with thread to form a chain and tested in a 100 cc. chamber containing modified Van Dyke-Hastings solution (16). The lever was maintained at a ratio of 12:1 so that the pen magnified the muscle excursion 12 times. The time necessary for the reactions to take place with this method is considerably longer than with other isolated smooth muscle reactions and the dose of all drugs, both stimulant and depressant, are also larger.

The addition of euphorbia to the bath causes an immediate relaxation of a normal untreated tracheal chain.

Histamine. As seen in Fig. 3, 1.0 cc. of euphorbia will relax almost to the baseline a contraction caused by 1.0 cc. of histamine (1.0 mg./cc.). After washing, the addition of euphorbia to the bath causes a definite relaxation of the muscle below the baseline. When histamine is then added to the bath before washing, the resulting contraction is less than one third as high as the contraction without euphorbia. After washing, the addition of histamine causes a contraction which approximates the first histamine contraction in height although it initiated from a lower baseline.

Caffeine (2.5 cc., 10 mg./cc.) will cause a relaxation of a histamine contraction; however, aspirin (2.0 cc., 5.0 mg./cc.) will not. A combination of these doses of aspirin and caffeine causes a relaxation equal to that obtained with caffeine alone. An increase in the amount of aspirin to a ratio of 6:1 for the aspirin to the caffeine does not enhance the effectiveness of the caffeine. As shown in Fig. 4, a combination of euphorbia, aspirin, and caffeine relaxes a histamine contraction of the tracheal chain below the baseline. After washing, euphorbia relaxes a similar histamine contraction not quite to the baseline but the addition of aspirin and caffeine brings it below the baseline, thus showing that there is a small degree of synergism between euphorbia, aspirin, and caffeine.

Egg White. Guinea pigs were sensitized to egg white by intraperitoneal injection of 5.0 cc. of egg white, 25% in physiologically normal saline. After two weeks, tracheal chains were made from these guinea pigs and when 1.0 cc. of egg white was added to the bath a histamine-type contraction was elicited.

Washing does not cause a relaxation of an egg white antigen contraction. The further addition of egg white to the bath, either before or after washing, does not have any effect on the tracheal chain; the contraction cannot be repeated. Egg white added to the bath of a tracheal chain from a nonsensitive guinea pig has no effect.

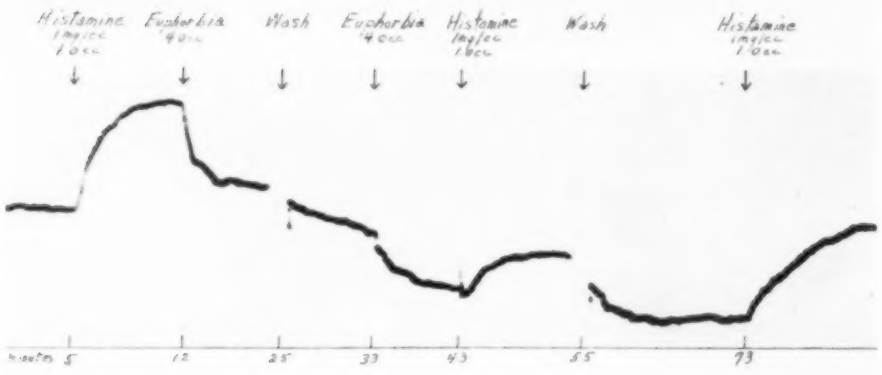

Fig. 3.—Relaxation and diminution by euphorbia of a histamine contraction of a guinea pig tracheal chain.

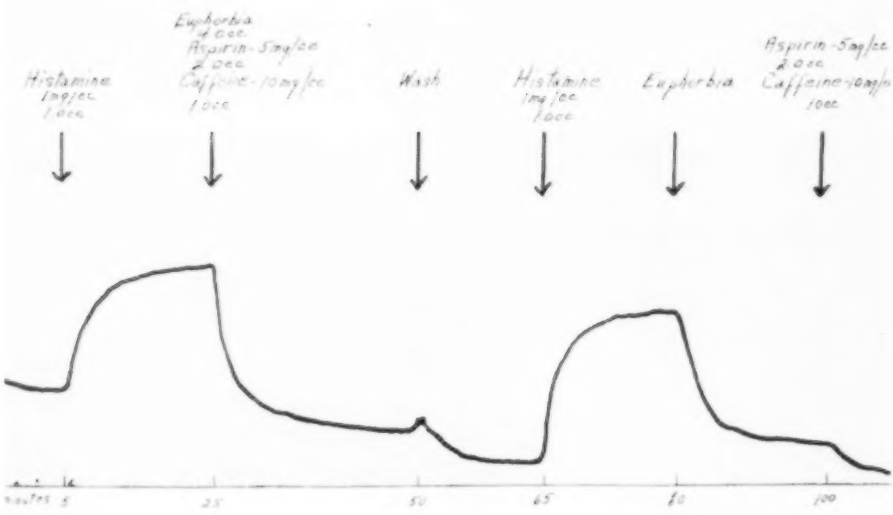

Fig. 4.—Relaxation of a histamine contraction of guinea pig tracheal chain by a combination of euphorbia, aspirin, and caffeine.

Figure 3 illustrates that 4.0 cc. of euphorbia will relax this contraction slightly, and the further addition of 4.0 cc. of euphorbia will relax the tracheal chain below the baseline. A state of contraction is not resumed following relaxation by euphorbia, even after washing. A histamine contraction can be elicited, thus showing that the chain is still active.

One cubic centimeter of caffeine (10 mg./cc.) will relax an antigen type spasm to the baseline. Two cubic centimeters of aspirin (5.0 mg./cc.) will not relax an antigen contraction; however, upon the addition of 1.0 cc. caffeine (10 mg./cc.) the chain relaxes to the baseline, and upon the addition of 4.0 cc. of euphorbia there is a relaxation far below the baseline. As shown in Fig. 6, when these same doses of euphorbia, aspirin, and caffeine are added simultaneously there is an immediate relaxation below the baseline, comparable to that achieved when the three drugs are added separately.

The work of Castillo (15), using horse serum as the antigen in sensitizing guinea pigs, was confirmed. The effect of euphorbia on a horse serum antigen contraction of the tracheal chain was comparable to the above work using egg white.

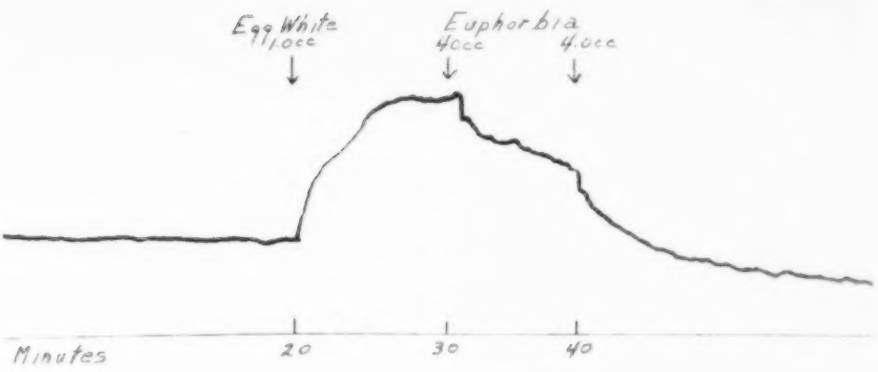

Fig. 5.—Relaxation by euphorbia of an egg white contraction of a guinea pig tracheal chain.

DISCUSSION

In order to evaluate the relative potency of euphorbia as an antispasmodic, and particularly as a bronchodilator drug, it appears desirable to establish certain basic relationships between typical smooth muscle such as guinea pig ileum and the more specialized tracheal musculature. Some of these more basic relationships can be illustrated as follows:

Castillo and deBeer (10) showed that bronchodilator drugs such as epinephrine, aminophylline, and papaverine will relax the uncontracted tracheal chain whereas Benadryl will not. The present studies show that euphorbia and caffeine also will relax the normal tracheal chain although neither has a prominent depressant action on normal guinea pig ileum. This action of euphorbia and caffeine on the trachea has been shown to be reversible.

A marked difference in the sensitivity to histamine is exhibited by the tracheal chain and by the ileum. A concentration 500 times as great as the ileum concentration is required to induce a contraction of the trachea. This suggests a correlation between the depressant action of euphorbia on these uncontracted tissues and on the histamine contraction since only four times the ileum dosage of euphorbia is required to relax the histamine-induced tracheal contraction. A comparable correlation exists with caffeine since this agent will relax a histamine contraction of the tracheal chain but not a histamine-induced contraction of the ileum. The same is true for aminophylline (10, 11, 12). A reverse correlation appears to be true with aspirin since this drug will relax histamine contractions of the ileum but not histamine contractions of the trachea. It is interesting that this reverse correlation also exists in certain spasmolytic agents such as Trasentin and Syntropan (10, 11, 13, 14). When aspirin is combined with either caffeine or euphorbia it does not interfere with the relaxant action of these drugs on the histamine contraction of the trachea.

Another factor requiring careful evaluation is the relationship between histamine induced and antigen induced contractions of the isolated tracheal chain from normal and sensitized guinea pigs. It has been shown, both in these studies and by others (10, 15), that normal and sensitized tracheal chains are approximately equal in sensitivity to histamine. On the other hand, an allergen such as horse serum or egg white will induce contractions only in sensitized trachea. Castillo (15) has shown that Benadryl 1:15,000,000 will relax a histamine contraction of the trachea but that antihistamines such as Benadryl, Pyribenzamine, or Neo-Antergan in concentrations

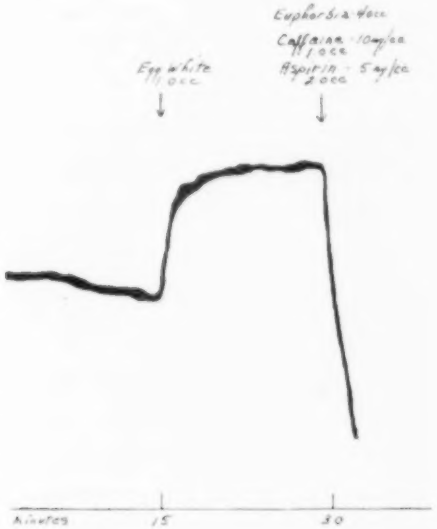

Fig. 6.—Relaxation by a combination of euphorbia, aspirin and caffeine of an egg white contraction of the guinea pig tracheal chain.

of 1:250,000 will not effectively relax an anaphylactic contraction. On the other hand, bronchodilator drugs such as epinephrine, aminophylline, and papaverine will effectively relax such a contraction. The present studies indicate that euphorbia in a concentration only twice that required for relaxing histamine spasm will relax an antigen-induced spasm. Castillo (15) and Castillo and deBeer (10) have shown a ratio of 4:1 for epinephrine under these conditions (1:5,000,000 for allergen; 1:20,000,000 for histamine). While the present studies show that caffeine effectively reduces the antigen-induced spasm of the trachea, the effective dosage ratio between the histamine-induced and antigen-induced spasm was not established.

The results of these studies on the isolated tissues appear to correlate well with the studies of Landau, *et al.* (8), on intact animals. These authors established the 100% protective antihistamine dose of Benadryl and Pyribenzamine in guinea pigs. To obtain 100% survival from anaphylactic shock, it was necessary to increase the antihistamine dose of Benadryl five times (from 0.6 to 3.0 mg./Kg.), and of Pyribenzamine 100 times (from 0.01 to 1.0 mg./Kg.). They point out that some of the antihistamines are more effective than others in protecting against histamine and that the ratio of the dosage required to protect against anaphylactic death is greater when the histamine protective dose is very small.

In summary, these experiments demonstrate that, in addition to the general myotropic antispasmodic properties previously reported (6), euphorbia exerts a relatively specific depressant action on tracheal musculature. In this respect it differs from antihistamines and antispasmodics of the atropine type and resembles epinephrine and aminophylline. Similar properties are demonstrated for caffeine, while aspirin neither shares nor interferes with these properties. The actions of euphorbia and caffeine are synergistic to some extent. Careful evaluation of the similarity between the action of these drugs and that of epinephrine and aminophylline, particularly on histamine and allergic contractions of the trachea, provides an experimental basis for the observed clinical efficacy of a combination of euphorbia and caffeine.

CONCLUSIONS

1. The partially purified dealcoholized fluidextract of *Euphorbia pilulifera* is effective in relaxing contractions of isolated mouse ileum, guinea pig ileum, and guinea pig uterus produced by acetylcholine, barium chloride, and histamine, but is only mildly depressant to the normal muscles.

2. This preparation is effective in relaxing contractions of guinea pig tracheal chains by histamine.

3. This preparation will relax contractions by egg white antigen in sensitized animals.

4. This preparation relaxes the normal tracheal musculature but not visceral smooth muscle.

5. Similar effects are demonstrated for caffeine, but not for aspirin.

6. When evaluated on the basis of comparative ratios, the action of the fluidextract of euphorbia on both normal and sensitized trachea appears to be relatively specific.

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Use of the Trypan Blue and Rabbit Eye Tests for Irritation*,†

By JAMES O. HOPPE, EVELYN B. ALEXANDER, and LLOYD C. MILLER

An objective method has been described for determining the irritant properties of substances designed for parenteral administration by means of the trypan blue test. Various concentrations of the substance to be studied are injected intracutaneously into the abdominal skin of the rabbit followed by the intravenous injection of 10 mg./Kg. of trypan blue. Extravasation of the blue dye into the injured areas is interpreted according to a table of weighted numerical scores. The test results are summarized in terms of the Threshold Irritant Concentration (T. I. C.) defined as the concentration expressed in per cent which produces no more than a "mild" irritation. The mucous membrane test for irritation using the rabbit eye (9) has been extended to include a simplified and more practical method for expressing the results in terms of the T. I. C. The trypan blue response of quinine hydrochloride was not abolished by an antihistaminic drug. Histamine diphosphate failed to produce a typical trypan blue response in rabbits whereas histamine wheals in the dog stained a deep blue following the intravenous injection of trypan blue.

LOCAL tissue irritation often plays an important role in the evaluation of the pharmacological properties of a new drug designed particularly for parenteral administration or mucous membrane application. Even though a compound may be extremely promising from a pharmacological standpoint, its irritant properties may be so great as to preclude its clinical use. It is desirable, therefore, to have available a test capable of measuring irritation in experimental animals with sensitivity sufficient to exclude compounds which are potentially injurious to tissue, yet not overlook those which might be nonirritating at concentrations within the useful therapeutic range. Such a test should embrace not only the elements of ease of performance, objectivity, reliability of interpretation and replication, but also a simplified method for expressing the results obtained in terms having practical significance.

The trypan blue test for irritation is based on the observation that intravenously injected colloidal dyes promptly seek out and stain injured skin areas as a result of an increased vascular permeability (1-3). In 1937, Tainter, Throndson, and Lehman (4) utilized this phenomenon in their investigation of the irritant properties of sodium bisulfite solutions. Various concentrations of bisulfite solution were injected subcutaneously in the previously shaved abdominal skin of the rabbit followed by the intravenous injection of 10 mg./Kg. of trypan blue. The results were reported in qualitative terms. Three years later, Weatherby (5) described a similar technique for the quantitative estimation of chemical irritation by subcutaneous injection of the test solutions into the dorsal surface of the rabbit ear. The intensity of the stained areas was compared with standard color scales. However, the abstruse method of reporting the results leaves much to be desired from a practical standpoint. While not primarily interested in the measurement of irritation, Rocha e Silva and Dragstedt (6) employed the trypan blue test in their investigation of the effect of histamine and substances capable of liberating histamine upon intracutaneous injection into the abdominal skin of rabbits. Last and Loew (7) likewise used the trypan blue test to study the effect of antihistamine drugs on increased capillary permeability following intradermal injections of histamine and related substances in rabbits. Both of these last groups of investigators recorded their results as 0 to + + + +.

In a study of the acid-base tolerance of the cornea, Friedenwald, Hughes, and Herrmann (8) developed a numerical system for recording the objective measurement of injuries to rabbit eyes. Draize, Woodard, and Calvery (9) have modified and extended this principle of assigning numerical values to similar physiological phenomena. Their scheme for measuring mucous membrane irritation adequately fulfills the requirements of simplicity, objectivity, and reliability of interpretation and replication. However, instead of leaving the results in abstract numbers, it seems reasonable to assume that they could be divided into arbitrarily selected ranges with appropriate adjective equivalents. Since minimal irritation is of primary importance in the selection of new drugs for clinical trial, the expression "Threshold Irritant Concentration" (T. I. C.) has been adopted to represent the

* Received May 29, 1948, from the Biology Division, Sterling-Winthrop Research Institute, Rensselaer, N. Y.
† The authors gratefully acknowledge the helpful suggestions and guidance of Dr. M. L. Tainter throughout this work.

minimum percentage concentration which may be expected to produce not more than a "mild" irritation.

The present communication describes a method for the objective measurement of irritation by means of the trypan blue test. A simplified method is offered for expressing the results of both the trypan blue and rabbit eye irritation tests in practical terminology. Several examples are included.

METHOD

Substances studied included three local anesthetics: tetracaine hydrochloride, dibucaine hydrochloride and procaine hydrochloride; two cationic detergents: cetyldimethylbenzylammonium chloride (WIN 356)[1] and N-dodecyl-N,N-dimethyl-3,4-dichlorobenzylammonium chloride (WIN 1275);[2] an antihistaminic drug: N,N-dimethyl-N'-(3-thenyl)-N'-(2-pyridyl)ethylenediamine hydrochloride (WIN 2848);[3] quinine hydrochloride and ethanol. All solutions were prepared in 0.9% normal saline.

Albino rabbits, sex at random, weighing 2 to 3 Kg., were used throughout. For the trypan blue tests, the rabbits were fastened securely in a supine position and the hair carefully clipped from the abdominal area with a fine-bladed clipper. Clipping the rabbits before the day of the test offered no particular advantage since the regrowth of hair was often sufficient to interfere with interpretation. The bare abdominal surface was marked off into 6 or 8 areas of approximately 20 sq. cm. each by means of a soft wax pencil. In preparing solutions for injection, the highest concentration was prepared first and subsequent dilutions made therefrom in ratios of 1/2, 1/4, 1/8, 1/16, etc., in order to find the submaximal irritation range. A volume of 0.3 cc. of each dilution was injected intracutaneously into randomly designated areas of the abdominal skin of the rabbit. The injections were completed in approximately ten minutes. A dose of 1.0 cc./Kg. of a 1% solution of trypan blue in normal saline was injected intravenously ten to twenty minutes after the last intracutaneous injection of test solution. The sites of injection were examined at one-half, one, and three hours after injection of the trypan blue. The results were scored and the T. I. C. obtained in accordance with the directions given in Table I.

For the rabbit eye irritation tests, the rabbits were placed in ventilated boxes with only the head free. Solutions were prepared as described above and a volume of 0.2 cc. of solution was instilled into the conjunctival sac of one eye, leaving the other as a control. The lower lid was retracted gently and held away from the cornea for one minute, thus insuring exposure to the solution. The medicated eye was examined in comparison with the control eye at one, two, four, and eight hours after medication and the results scored according to the method of Draize, *et al.* (9). The T. I. C. was calculated from Table II.

As a rule, tests were made on 5 rabbits at each of 2 or more concentrations, each succeeding concentration being twice that of the immediately preceding concentration. The irritation scores were averaged for the respective concentrations at each reading to obtain the maximum average irritation score for each concentration occurring during the test period.

The results obtained by means of the trypan blue and rabbit eye tests are shown in Tables III and IV, respectively.

That the concentrations tolerated by intracutaneous injection into the skin of the rabbit are less than those tolerated by the mucous membrane is indicated by the smaller T. I. C.'s obtained in the trypan blue test. The 2 cationic detergents, however, appeared to be equally irritating by both mucous membrane application and parenteral administration. In the comparison of the 3 local anesthetics, the order of irritation from least to greatest was: procaine, tetracaine, and dibucaine by both methods of testing. The T. I. C.'s found for each of these local anesthetic drugs were in general agreement with the recommended concentrations given in N. N. R., 1948 (10). The T. I. C.'s for the antihistaminic drug, WIN 2848 were larger than those for quinine hydrochloride by both tests. Ethyl alcohol, in concentrations up to 5%, produced only a mild irritation by intracutaneous injection while concentrations up to 20% could be applied to the conjunctivas of the rabbit before a more than mild irritation resulted.

TABLE I.—NUMERICAL ESTIMATION OF THE INTENSITY OF STAINING BY TRYPAN BLUE AT THE SITE OF INJECTION OF AN IRRITANT SOLUTION IN RABBITS

No color	0
Faint but discernible blue color	2
Distinct blue color throughout	4
Deep blue color throughout	8
Ischemic central area surrounded by deep blue halo	16

Calculation of the Threshold Irritant Concentration[a]

Maximum Average Irritation Score	Adjective Rating
0	None
1–3	Mild
4–7	Moderate
8 or greater	Marked

[a] The Threshold Irritant Concentration (T. I. C.) is taken as that concentration expressed in per cent which produces no more than a mild irritation.

TABLE II.—CALCULATION OF THE THRESHOLD IRRITANT CONCENTRATION[a] FOR RABBIT EYE IRRITATION DATA OBTAINED BY THE METHOD OF DRAIZE, *et al.* (9)

Maximum Average Irritation Score	Adjective Rating
0	None
1–5	Mild
6–10	Moderate
11–15	Marked
16 or greater	Severe

[a] The Threshold Irritant Concentration (T. I. C.) is taken as that concentration, expressed in per cent, which produces no more than a mild irritation.

[1] Zetryn Chloride (Winthrop-Stearns Inc., trade name).
[2] Dymium Chloride (Winthrop-Stearns Inc., trade name).
[3] Thenfadil Hydrochloride (Winthrop-Stearns Inc., trade name).

TABLE III.—INTRACUTANEOUS IRRITATION BY THE TRYPAN BLUE TEST

Substance	Concentration in Per Cent	Maximum Average Irritation Score	Time in Hours to Max. Score	Adjective Rating	T. I. C.
Tetracaine HCl	1.0	16.0	1–3	Marked	0.5
	0.5	2.0	1–3	Mild	
	0.25	2.0	1–3	Mild	
Dibucaine HCl	0.40	5.6	1–3	Moderate	0.2
	0.20	3.6	1–3	Mild	
	0.10	1.6	1–3	Mild	
Procaine HCl	20.0	16.0	1–3	Marked	5.0
	10.0	16.0	1–3	Marked	
	5.0	2.0	1–3	Mild	
WIN 357	0.16	12.0	3	Marked	0.08
	0.08	3.6	3	Mild	
	0.04	3.6	1–3	Mild	
WIN 1275	0.16	10.8	1–3	Marked	0.04
	0.08	4.8	1–3	Moderate	
	0.04	2.0	1–3	Mild	
WIN 2848	2.0	16.0	1–3	Marked	1.0
	1.0	3.2	1–3	Mild	
	0.5	2.0	1–3	Mild	
Quinine HCl	1.0	8.8	3	Marked	0.25
	0.5	4.4	1–3	Moderate	
	0.25	2.4	3	Mild	
Ethanol	10.0	9.6	1–3	Marked	5.0
	5.0	3.6	1–3	Mild	
	1.0	2.0	1–3	Mild	

TABLE IV.—MUCOUS MEMBRANE IRRITATION BY THE RABBIT EYE IRRITATION TEST

Substance	Concentration in Per Cent	Maximum Average Irritation Score	Time in Hours to Max. Score	Adjective Rating	T. I. C.
Tetracaine HCl	2.0	10.0	8	Moderate	1.0
	1.0	4.8	2–8	Mild	
	0.5	2.8	8	Mild	
Dibucaine HCl	2.0	6.8	8–24	Moderate	0.5
	1.0	6.8	8	Moderate	
	0.5	4.8	8	Mild	
Procaine HCl	50.0	3.2	2–8	Mild	>50
	20.0	0.8	2–8	Mild	
WIN 357	0.2	8.4	8	Moderate	0.05
	0.1	6.4	1–8	Moderate	
	0.05	3.6	4–8	Mild	
WIN 1275	0.2	10.4	8	Moderate	0.05
	0.1	8.4	8	Moderate	
	0.05	3.2	8	Mild	
WIN 2848	10.0	6.4	8–24	Moderate	5.0
	5.0	2.8	1–8	Mild	
	2.5	2.8	2–8	Mild	
Quinine HCl	4.0	5.6	4–8	Mild	>4.0
	2.0	4.0	4–8	Mild	
	1.0	0.8	1–8	Mild	
Ethanol	80.0	7.2	2–4	Moderate	20.0
	40.0	6.0	2–4	Moderate	
	20.0	2.0	8	Mild	

TABLE V.—EFFECT OF WIN 2848, AN ANTIHISTAMINIC DRUG, ON THE TRYPAN BLUE RESPONSE OF RABBITS TO QUININE HCl

Concentration in Per Cent	Untreated Rabbits: Maximum Average Irritation Score	Untreated Rabbits: Adjective Rating	Treated Rabbits (10 Mg. Kg. of WIN 2848 S. C. 30 Min. before Trypan Blue Test): Maximum Average Irritation Score	Treated Rabbits (10 Mg. Kg. of WIN 2848 S. C. 30 Min. before Trypan Blue Test): Adjective Rating
1.0*	11.2	Marked	10.4	Marked
0.5	6.0	Moderate	4.4	Moderate
0.25	2.8	Mild	1.2	Mild
T. I. C.	0.25%		0.25%	

* Five tests at each concentration.

Antihistaminic drugs have been shown to diminish the increase in capillary permeability which follows the injection of histamine (7). Further, the release of histamine or a histamine-like substance has been associated with skin injury (11). Therefore, it was of interest to investigate the effect of an antihistaminic drug upon the typical trypan blue response of a known irritant such as quinine hydrochloride. If the trypan blue response, shown in Table III, resulted from the release of histamine, then pretreatment with an antihistaminic drug should prevent the staining of the irritated area. Five tests were made on 5 rabbits at concentrations of 1.0, 0.5, and 0.25% in normal saline. A second group of 5 rabbits received 10 mg. /Kg. of the antihistaminic drug, WIN 2848, subcutaneously, and thirty minutes later was subjected to the same procedure as the first group. The comparative results are shown in Table V.

It will be noted that while the maximum average irritation scores for each concentration were slightly less in the pretreated rabbits, there was not enough difference to change the adjective ratings for any of the concentrations and the T. I. C. in both tests agreed with that shown in Table III.

Each of the substances listed in Table III produced a wheal approximately 20 mm. in diameter and the staining, when it occurred, was confined to the wheals. With each, an optimum dose range could be found wherein changes in concentration could be discriminated by the intensity with which the site of injection was stained with trypan blue.

DISCUSSION

While it is difficult to eliminate subjective bias entirely in the interpretation of trypan blue irritation results, the assignment of numerical values to the different intensities of staining serves to transform qualitative observations into reasonably quantitative data with some degree of objectivity. Weighting the scores as given in Table I was based on the assumption that the response of a tissue to injury follows a geometric rather than an arithmetic progression.

The concentration relationship of each compound was fairly well graduated in the rabbit eye irritation test whereas abrupt changes from maximum average irritation scores of 10 to 2 or 3 were obtained in several instances upon halving the test concentration in the trypan blue test. These abrupt changes from marked irritation at one concentration to mild irritation at the next lower concentration appeared to be associated with a distinction between reversible and irreversible irritation phenomena. When the wheal at the site of injection was evenly stained there was no obvious evidence of tissue damage, but the presence of an ischemic area surrounded by a blue halo frequently was followed by necrosis and sloughing. Weatherby (5) also observed this blurring effect and attributed the appearance of the pale, central area to local tissue destruction.

Last and Loew (7) obtained positive trypan blue reactions following the intradermal injection of histamine diphosphate in rabbits. They described a halo staining pattern with concentrations of 0.1 to 1.0 mg. /cc. of histamine diphosphate and showed that antihistaminic drugs would diminish the trypan blue response. On the other hand, Darsie and coworkers (12), in a study of the histamine whealing response of various animals, found that rabbits were unsatisfactory. They reported negative whealing responses to the intradermal injection of histamine diphosphate in guinea pigs, rabbits, and cats, and positive responses in the dog, goat, and man. In the present investigation, intracutaneous injection of histamine diphosphate in concentrations from 0.1 to 10 mg. /cc. into the abdominal skin of albino rabbits produced no true wheals. The site of injection became flat within a few minutes after injection and remained as a circular blanched area 20 to 30 mm. in diameter for two to three hours. Staining of the blanched areas by injected trypan blue was not observed at any of the doses of histamine employed. In one or two instances a diffuse pale blue coloration appeared around the blanched areas but this phenomenon could not be consistently reproduced.

In contrast, the same concentrations of histamine diphosphate injected intracutaneously in the dog produced distinct wheals approximately 30 mm. in diameter which became deeply stained upon the intravenous injection of 10 mg. /Kg. of trypan blue. Intracutaneous injection of similar concentrations of quinine hydrochloride in the dog produced wheals which were smaller in diameter and slightly more elevated than those produced by histamine diphosphate. The intensity of the blue color in the quinine wheals in the dog varied with the concentration whereas the intensity of the bluish coloration in the histamine wheals varied but slightly with concentrations of 0.1 to 10 mg. /cc. The injection of histamine diphosphate produced a vigorous pain response in both the dog and the rabbit.

It would appear that the characteristic trypan blue response to the intracutaneous injection of solutions of irritant substances in rabbits is not due solely to the release of histamine. It seems more reasonable to assume that an increase in capillary permeability resulting from the intradermal injection of an irritant compound to an extent permitting extravasation of trypan blue may be a fundamental property of the molecule. Whether the substance produces its irritant effect physically or chemically through the production of a mediator other than histamine remains for future investigation to clarify.

When considering the irritant properties of a given compound in solution, it is customary to think adjectively in terms of "mildly" irritating or "moderately" irritating. However, these terms are without meaning unless associated with some method of objective measurement from which they can be derived. The use of the expression Threshold Irritant Concentration readily brings to mind the percentage concentration of a substance which may be expected to produce a "mild" irritation, thus associating adjective description with concentration without sacrificing objectivity.

Neither the trypan blue nor the rabbit eye test for irritation gives any information on the subjective symptom of transient stinging or smarting frequently observed in clinical trial. If a substance is sufficiently painful to cause an observable pain response when injected, it usually will produce flagrant evidence of irritation by the procedures described here. Histamine, however, appears to be an exception since it produces a marked pain response at concentrations of 10 mg. /cc. or less without producing evidence of permanent tissue damage.

SUMMARY

An objective and reasonably quantitative method for estimating intracutaneous irritation by means of the trypan blue test has been described. A scheme for expressing the results of both the trypan blue and rabbit eye irritation tests is presented. The common denominator is the percentage concentration which produces no more than a mild irritation for which the expression Threshold Irritant Concentration (T. I. C.) was suggested.

Intradermal injections of histamine diphosphate did not produce typical wheals in the rabbit. The site of injection in the rabbit skin was characterized by a circular blanched area which failed to demonstrate a blue color following the intravenous injection of trypan blue. Intracutaneous injection of histamine diphosphate in the dog, however, produced characteristic wheals which stained blue following the intravenous injection of trypan blue. Pretreatment with an antihistaminic drug failed to diminish the characteristic trypan blue response of quinine hydrochloride in the rabbit.

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Phosphorus Poisoning in Waterfowl*

By DON R. COBURN, JAMES B. DeWITT, JAMES V. DERBY, Jr., and ERNEST EDIGER

Black and mallard ducks have been found to be highly susceptible to phosphorus poisoning. The results of an investigation of the effects of poisoning from the administration of white phosphorus are reported.

Losses of waterfowl with histories indicative of metallic poisoning have come to our attention on numerous occasions during the past few years. Scarcity of reports in the literature of such poisonings brought a realization that too little work has been done on the symptomatology, chemical analyses, and microscopic pathology necessary for differential diagnosis of such conditions.

Among poisoning cases received for diagnosis, the presence of phosphorus has frequently been suspected. Chemical analyses, however, have not always confirmed the pathological findings in such cases. Results of tests of tissues in some instances (1948) showed phosphorus in lower concentration than is found in tissues of normal birds. Losses in successive years from an area in which positive evidence of phosphorus poisoning was obtained (phosphorescence and visible smoking of gizzard contents) made it imperative that the disparity between characteristic pathological findings and the variable chemical analyses should be satisfied. For these reasons it was decided that tissues for histopathologic study and for chemical analysis from experimental cases would be the easiest solution to the problem. For a check on the possibility of loss of phosphorus from the tissues after death, as suspected in field specimens, provision was made for holding tissues for variable periods of time between death and chemical analysis.

PROCEDURE

Wild mallard and black ducks taken in the course of banding operations were selected on the basis of healthful appearance, uniformity of size, and an approximately even division of sexes. The birds were held for ten days to accustom them to the change in diet and pen confinement. Six birds selected for study of normal tissues were sacrificed at one time. Blood samples for the determination of hemoglobin content and cell counts were obtained by puncture of the wing vein. Samples were drawn on alternate days. Tissues for histopatho-

* Received June 28, 1949, from the Branch of Wildlife Research, Fish and Wildlife Service, U. S. Department of Interior, Patuxent Research Refuge, Laurel, Md.

logical preparations were taken at autopsy. The weight of tissues removed for this purpose was determined. The total weight of the heart, liver, and kidneys was then computed and the organs saved for chemical analysis. The latter were held in the refrigerator at 4.0° for different periods of time. At the end of the predetermined holding periods, the samples were placed in deep freeze storage and held until removed for analysis.

These tissues were prepared for analysis according to the procedure of the Association of Official Agricultural Chemists (1) and the total phosphorus content determined by the modified A. O. A. C. molybdenum blue method developed by Stoloff (2). In addition to hematoxylin eosin stained tissue preparations, frozen section preparations of the liver were stained with nile blue, osmic acid, and sudan IV with a counterstain of hematoxylin, for the demonstration of fat.

Dosage Levels

To provide comparative material of both acute and chronic degrees of poisoning 24 birds were administered doses of 12, 6, 3, 1½, and 1.0 mg. of white phosphorus in almond oil per Kg. of body weight.[3] Doses were measured with a syringe and delivered directly into the gizzard by the use of a 10-inch flexible catheter. The smaller dosage groups were run in duplicate so that birds were dosed both daily and on alternate days until death resulted or for a period of two weeks. Survivors at this time were sacrificed for tissues.

RESULTS OF CLINICAL AND HISTOPATHOLOGICAL STUDIES

Symptomatology

Acute Poisoning. Marked variation in survival time was shown in both acute and chronic poisonings (Table I). A chance inclusion of tamed ducks, in the test group receiving the higher dosage levels, appears to have introduced a death rate factor connected with nervous excitability. These birds (620 series) had been used on an unrelated study which required almost daily handling for several weeks. This handling had made actual pets of the birds much in contrast with the remainder of the group which were wild, untamed birds. The four tame birds showed early symptoms of distress with inability to swallow, following dosing. They, however, lived longer than any other bird dosed at the same level, received a dose on the second day, and all four died within thirty-three hours of the initial dose. Lack of nervous excitability in the tame birds may explain the delay of nervous symptoms associated with terminal convulsions.

The longest period of survival for any untamed bird receiving a single dose of 3 or more mg./Kg. P was seventeen hours. Two birds which received 6 mg./Kg. P and one receiving 12 mg./Kg. P lived sixteen hours. Shortest survival time (three and one-half hours) was for one bird which received 12 mg./Kg. P. One bird which received a single dose of 3 mg./Kg. P lived only six hours.

[3] It is possible that the rate of absorption of the phosphorus and its physiological activity may have been affected by the almond oil used as a solvent. No evidence on this point is available.

The first symptoms observed were those of depression and in a few instances an increased thirst with inability to swallow. These symptoms developed within a period of two or three hours. Shortly thereafter leg weakness became apparent with the legs unable to support the body weight. This weakness progressed rapidly with the bird assuming a normal sitting position, the head held erect and swaying from side to side in a slow rhythmic motion. The eyes remained open and at no time was paralysis of the nictitans membrane observed in either acute or chronic cases. Attempts at handling at this stage often resulted in convulsive struggling ending abruptly in death. Birds observed in the death struggle, the scattered cage litter and the abnormal positions of birds found dead, showed that death in acute poisonings is preceded by violent convulsions.

Chronic Poisoning. Birds dying from chronic phosphorus poisoning (survival of five to ten days) become progressively less active and showed a steady loss of weight. Survival time and weight loss of individual birds showed marked variation. One of two birds receiving 1 mg./Kg. P daily died with symptoms of acute poisoning within seventy-two hours while the other survived ten daily dosings.

Paralysis of the gullet was observed in two cases of chronic poisoning as well as in several birds which died of acute poisonings. Development of the paralysis in two chronic cases was late in the decline of the birds and definitely a part of the cause of death.

Field cases are described as having an "appearance of being drunk," "ungainly flight," and "peculiar progress on the ground with stumbling and loss of balance." Acute poisoning has always predominated in the cases submitted for diagnosis.

Postmortem Findings

Acute Poisoning. Dark, pulpy, congested livers and kidneys, characteristic of acute phosphorus poisoning, were the most consistent lesions found. Subcutaneous tissues showed little change from normal. Visceral organs in most instances showed congestion. Small areas of necrosis of the heart muscle were observed in a few instances. Duodenitis of a mild character was occasionally observed. Congestion of the spleen was not constant. The livers in two cases showed light yellow discoloration outside of the range of normal. With these two exceptions there was marked congestion and great friability of the liver in every acute poisoning case. The amount and appearance of the bile did not show abnormal alteration.

Chronic Poisoning. Birds which lived more than seventy-two hours following ingestion of phosphorus showed loss of weight. Such early loss was represented chiefly by reduction of the fatty tissues, both skeletal and visceral, and to a reduced amount of material in the gastrointestinal tract. The greatest loss of weight recorded in our experimental birds was 320 Gm. (initial weight, 875 Gm.). This bird received a total of 7.9 mg. phosphorus, administered in 6 doses at the 1½ mg./Kg. level (based on the initial weight), and was active when sacrificed on the tenth day. The tissues of birds of this period of survival appeared anemic. Icterus was never prominent. Mesenteric blood vessels in both chronic and acute cases showed varying degrees of in-

TABLE I.—SUMMARY OF ANALYSES OF DUCK TISSUES

Group	Band No.	Dosage Level, Mg./Kg.	No. of Doses	Total P Administered, Mg.	Survival Period	Total P Content of Tissues: Liver	Heart	Kidney
Control	Norm. Mall.	0	0			0.249	0.108	0.123
	716,033					0.270	0.138	0.130
	716,089 M, d.					0.175	0.160	0.214
	716,032 B, d.					0.390	0.222	0.243
	716,035 B, d.					0.264	0.123	0.226
	716,038 B, h.					0.288	0.171	0.262
	716,028 B, d.					0.181	0.191	0.238
	716,025 M, d.					0.327	0.159	0.171
					Av.	0.245	0.159	0.197
	716,036 M, d.	1.0	4	1.4	3 days	0.145	0.195	0.219
	716,026 B, d.	1.0	10	10.7	14 (K)	0.445	0.207	0.280
	716,001 M, d.	1.0[a]	6	6.5	18 (K)	0.179	0.305	0.321
	716,011 B, h.	1.0[a]	6	4.9	14 (K)	0.164	0.270	0.313
					Av.	0.158	0.244	0.283
2	716,037 B, d.	1.5[b]	3	4.4	4.5 days	0.180	0.195	0.232
	716,027 B, d.	1.5[b]	6	7.9	10 (K)	0.196	0.236	0.200
	716,013 B, h.	1.5[b]	2	2.95	1.5 days	0.222	0.260	0.271
	716,030 B, d.	1.5	10	15.0	14 (K)	0.223	0.227	0.189
	716,007 M, d.	1.5[b]	4	6.0	7 days	0.150	0.210	0.249
	716,005 B, d.	1.5[b]	6	8.0	14 days	0.186	0.198	0.288
					Av.	0.194	0.224	0.238
3	716,016 B, h.	3.0	1	3.0	6 hr.	0.179	0.175	0.180
	716,020 B, h.	3.0	1	3.0	17 hr.	0.178	0.229	0.211
	629,707 M, h[c]	3.0	2	6.0	33 hr.	0.175	0.279	0.309
	629,706 M, h[c]	3.0	2	6.0	33 hr.	0.296	0.267	0.191
	629,715 M, d[c]	3.0	2	6.0	33 hr.	0.110	0.267	0.112
	629,719 M, h[c]	3.0	2	6.0	33 hr.	0.317	0.201	0.246
					Av.	0.209	0.236	0.208
4	716,034 B, d.	6.0	1	6.0	8.5 hr.	0.114	0.174	0.188
	716,039 M, h.	6.0	1	6.0	16 hr.	0.197	0.224	0.279
	716,031 B, d.	6.0	1	6.0	4.5 hr.	0.161	0.226	0.193
	702,720 M, d.	6.0	1	6.0	20 hr.	0.329	0.263	0.281
					Av.	0.200	0.222	0.235
5	716,019 B, h.	12.0	1	12.0	16 hr.	0.166	0.209	0.164
	716,029 M, h.	12.0	1	12.0	8.5 hr.	0.171	0.184	0.171
	716,014 M, d.	12.0	1	12.0	3.5 hr.	0.196	0.229	0.260
	716,010 B, h.	12.0	1	12.0	4 hr.	0.216	0.194	0.274
					Av.	0.187	0.204	0.217
Redheads	Md. Game Comm.							
	No. 1, Female					0.139	0.180	0.201
	No. 2, Male					0.141	0.172	0.155
	No. 3, Male					0.133	0.201	0.139
	No. 4, Female					0.085	0.335	0.187
					Av.	0.124	0.222	0.168

M, d.—Mallard drake; B, h.—duck hen; K—sacrificed.
[a] Irregular dosing. [b] Alternate daily dosing. [c] Tamed ducks.

jection. The lungs in most cases appeared normal. The liver in some cases showed a light color commonly described as characteristic of phosphorus poisoning though typical yellow atrophy was not observed in any case. The spleen and kidneys showed some enlargement of the former organ and moderate to marked hypertrophy with congestion in the latter.

Field Cases. In contrast with the findings in experimental cases, a picture of greater change can be described for poisoned birds collected in the wild. The gastrointestinal tract of most birds submitted for diagnosis showed well-filled gizzards and fairly normal content in the posterior portion of the tract. The duodenum in most instances has shown severe inflammatory change with hemorrhage, necrosis, and sloughing characteristic of the corrosive action of elemental phosphorus. The kidneys of all specimens have shown marked congestion with a pulpy consistency and easy rupturing. In no case did the condition of the organs resemble that found in the experimental birds dying as the result of chronic poisoning. The causes for this dissimilarity are undetermined. It is possible that the field specimens died from the effects of ingesting a single massive dose of elemental phosphorus, or that the almond oil used as a solvent in the experimental studies may have affected the action of phosphorus on the tissues. Lung tissue impressions have not shown changes in shape of the erythrocytes as is found in the chronic experimental cases.

Microscopic Pathology

The classical cellular changes described in phosphorus poisoning, in general, were found in both acute and chronic cases. Fatty degeneration of the liver and kidneys was quite constant. The degree in which these changes were present seemed to depend upon the size of the dose and the period

of survival. The most pronounced changes were found to have occurred in birds which died between the third and tenth days. In cases which survived beyond the fourteenth day there was evidence of repair in the liver. Four birds which were sacrificed on the fourteenth day showed marked damage (similar to that shown in Figs. 2 and 3) to both liver and kidneys. Acute cases showed multiple hemorrhages and extensive necrosis in the kidneys.

Associated with the degenerative change in the liver there was observed in a few birds a condition which is not commonly described in connection with this type of poisoning. Liver cells in which the cytoplasma has been replaced with hemosiderin, while retaining comparatively normal outlines and intact nuclei, were frequently found in the sinusoids, bile ducts, and veins of the liver. In numerous instances in the chronic cases, multi-nucleate parenchymatous liver cells and cells with greatly enlarged nuclei, though otherwise normal in appearance, were observed in the more nearly intact abnormal liver tissue. The degree of degeneration shown by other cells in the vicinity leaves these former changes open for speculation as to whether they represent degenerative or regenerative processes. It is believed by the writers that in most instances the irregularly nucleated cells represent reparative processes.

Demonstration of the fat deposits obtained with different stains appeared to be dependent upon the rate and/or stage of the cellular change. In cases of acute poisoning where the fat droplets are not large, they were well defined with sudan IV. In cases succumbing after three or more doses, with the fatty degenerative change well advanced, the nile blue stain revealing various fatty acids, with diffuse staining as well as discrete fat droplets, proved to be the best of the three stains used. In the cases of chronic poisoning, osmic acid gave the sharpest differentiation of the smaller fat globules observed at this stage.

BLOOD CELL CHANGES OBSERVED IN EXPERIMENTAL CHRONIC PHOSPHORUS POISONING OF DUCKS

While not concerned in diagnostic procedures the blood dyscrasia observed serves in illuminating other less clear tissue changes. Phosphorus poisoning produces a change and shift in the blood picture roughly comparable with those resulting from lead poisoning. The true direction of either has not been determined with certainty and possibly is not evident in a single blood sample.

Of cellular changes in the blood resulting from repeated phosphorus intake, the most marked response is exhibited by the red cells. A peak in the proportion of juvenile to mature forms is first attained, to be followed, except for the intervention of death, by a gradual increase of the latter. Many abnormal mature forms of red cells appear and the percentage of these continues to rise, evolving, among other types, a blue staining cell which sometimes resembles a droplet of paint.

A pattern of progressive erythrocyte change observed in several chronic cases includes the initial pronounced therapeutic effect of stimulation of the hemopoetic system with its shower of immature cells. Coincident with this release there is an increase of damaged, mature erythrocytes.

As the release of increasingly less mature cells continues, forms resembling lymphocytes appear and misshapen, degenerate mature forms increase until a peak of response or imbalance is reached. In the decline, bizarre forms of both immature and mature cells appear including mitotic stages, sickling and warping of the former, and budding, bullouts, and folding forms in the latter.

Tracing the influence of the successive phosphorus doses on white cell ratios was complicated by abnormal white cell patterns in several of the ducks previous to dosing. A shift of leucocyte ratios occurs through the course of the treatment but neither is the range striking nor the pattern totally consistent.

White cell modification becomes evident at about the same time the juvenile red blood cell picture includes the extreme immature stage. The cytoplasm of some large lymphocytes take a deeper blue stain, heightening the resemblance to the early stage red cells.

Atypical basophiles are occasional. These show either a tendency toward irregular distribution of the granules, fewer granules, or granules variously stained through shades of faded blue, sometimes lavender, and less often pink. The basophile periphery may be prickled. Heterophile granule reduction in numbers is usual but variable in different preparations from the same ducks and also variable in full extent in different ducks. Weakening of the wall of heterophiles and the apparent relaxed turgidity of the large lymphocytes and

Captions For Figures 1–6

Fig. 1.—Kidney of experimental bird 716,036. Glomerulitis with loss of epithelial lining is shown. Necrosis of tubular epithelium was general throughout the kidney. Survival time, three days. Hematoxylin-eosin stain. ×400.

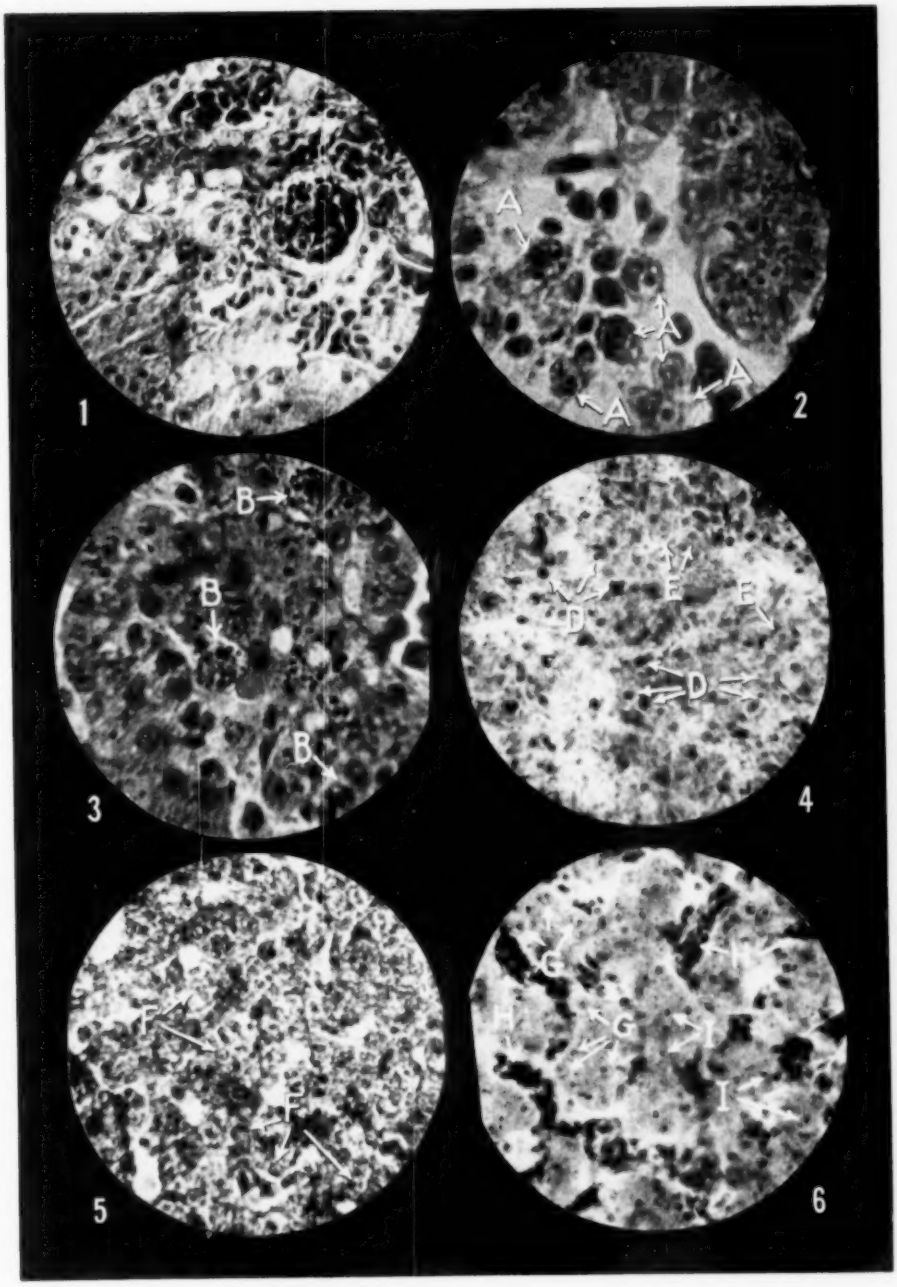
Fig. 2.—Liver of experimental bird 716,027. Degenerate liver cells (*A*) with blood constituents and debris in a large sinusoid. Sacrificed on tenth day. Hematoxylin-eosin stain. ×800.

Fig. 3.—Same specimen as Fig. 2. Necrotic liver cells (*B*) are seen in the process of facing shed from more nearly normal lobule into lymph space. Note mitotic figures and multinucleate liver cells. Hematoxylin-eosin stain. ×800.

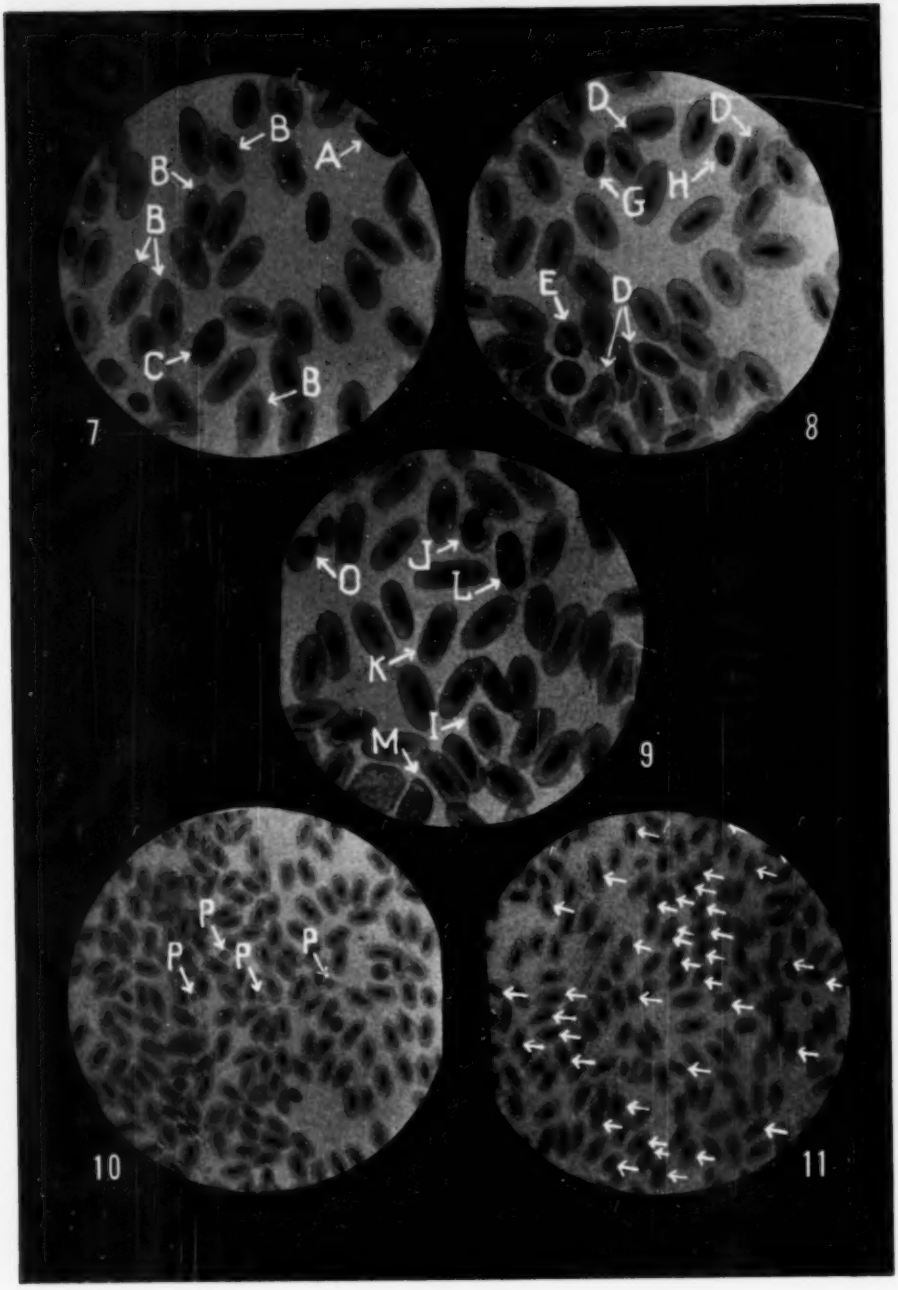
Fig. 4.—Liver of experimental bird 716,013. Discrete fat droplets are seen (*D*), and nuclei of cells (*E*), in this case of acute poisoning. Survival time, thirty-six hours. Sudan IV stain with hematoxylin counterstain. ×400.

Fig. 5.—Liver of field case 4. The diffuse deep staining fat in this necrotic liver, characteristic of acute poisoning, could not be mistaken. Nuclei of liver cells (*E*) have taken the hematoxylin counterstain. Sudan IV with hematoxylin counterstain. ×400.

Fig. 6.—Liver of field case 1. Fat droplets (*F*) are seen as very small points in this greatly congested liver. Necrosis is prominent but not as severe as in field case 4. Note scattered intact nuclei of liver cells (*G*). Also sinusoids packed with blood cells (*H*) the nuclei of which have taken the hematoxylin counterstain. Sudan IV stain. ×400.



Figures 1–6



Figures 7–11

Captions For

Figures 7–11

Fig. 7.—Duck 716,027. Blood specimen taken on eighth day after initial dose of 1½ mg./Kg. with total phosphorus 6.6 mg. given in 5 doses. An effect frequently observed is shown in the early stage juvenile erythrocyte (*A*). "Pointing" may be seen on some mature cells (*B*). The cell with the fragmented nucleus (*C*) is an expected form for this degree of poisoning. × 800.

Fig. 8.—Duck 629,719. Specimen prepared twenty-six hours after single dose, 3 mg./Kg. Erythrocyte susceptibility to mechanical distortion, prominent in advanced chronic and in acute poisoning, is seen (*D*). Degenerative change toward formation of curious "paint" cell shown by three cells (*E, G, H*). × 800.

Fig. 9.—Duck 716,026. Blood specimen taken on eighth day after initial dose of 1 mg./Kg. with total phosphorus 5.4 mg. given in 5 doses. Range in forms of juvenile erythrocyte (*I, J, K, L*) and resemblance to large lymphocytes (*M*) is shown. Mechanical impressibility of lymphocyte wall is evident. Deformation of erythrocyte by constriction (*O*) or yeastlike "budding" is surpassed only by "pointing" prior to pressure susceptibility. × 800.

Fig. 10.—From same specimen as Fig. 7. Increase of non-nucleated erythrocyte (*P*) closely related to period of juvenile cell influx appears in advanced chronic or acute poisoning. This photograph also shows two "paint" cells and pressure distortion of erythrocytes. × 400.

Fig. 11.—From same specimen as Fig. 8. The increased juvenile erythrocyte content of this field is apparent, though all are not clearly differentiated (arrows). Juvenile erythrocytes at this stage of poisoning constitute approximately 25 to 30% of the total red cells. × 400.

monocytes becomes evident by breaking and spilling in the former and by indentation, wrinkling, and "swiped" distortion in the latter. Shrinking of small-type lymphocytes and increased density or darkening of the nucleus makes differentiation from platelets difficult.

RESULTS OF CHEMICAL ANALYSES

Previous studies (1948)[2] had shown that the phosphorus content of livers from normal ducks was appreciably higher than that of livers from birds dying as the result of supposed phosphorus poisoning. The data were insufficient to permit definite conclusions, but it appeared that chemical analysis of tissues might offer a diagnostic test for the detection of phosphorus poisoning. Such a test would supplement findings by histological examination. The present series of tests was conducted in order to explore this possibility.

[2] Unpublished data.

RESULTS AND DISCUSSION

All analytical findings are summarized in Table I. Since this table is bulky, interpretation may be facilitated by considering various component parts.

Comparison of Normal and Pathological Tissues.—The average values (total phosphorus content) obtained in all analyses were:

Group	Total Phosphorus Content, %: Heart	Liver	Kidney	Muscle
Normals	0.159	0.245	0.197	0.143
Pathologicals	0.227	0.192	0.234	0.180

The phosphorus content of the liver was reduced, and that of the heart and kidneys was increased as a result of the ingestion of elemental phosphorus. When the data were analyzed statistically, it was found that all differences were significant. (The values for "P" were: Heart, less than 0.01; kidney, 0.01; liver, 0.03.) The number of samples of muscle tissue was too small to permit statistical analysis of the data. However, the average value of muscle was 26% higher in the case of the pathological tissues.

Having established that a significant difference is to be found in the total phosphorus contents of normal and pathological and normal tissues, we may now examine the effects of different dosage levels. First, it may be seen that the acute toxic level lies between 1.5 and 3.0 mg./Kg. All birds receiving 1.5 mg./Kg. P survived several days, and received several doses, while all birds receiving 3 mg./Kg. P died within a few hours after dosing. The analytical findings were:

Group	Total Phosphorus Content, %: Heart	Liver	Kidney
Normals	0.159	0.245	0.197
Pathologicals, chronic	0.232	0.180	0.256
(*t*)[3]	3.549	2.973	2.564
(P)	−0.01	−0.01	−0.01
Pathologicals, acute	0.223	0.200	0.219
(*t*)	3.978	1.662	0.939
(P)	−0.01	+0.01	0.35

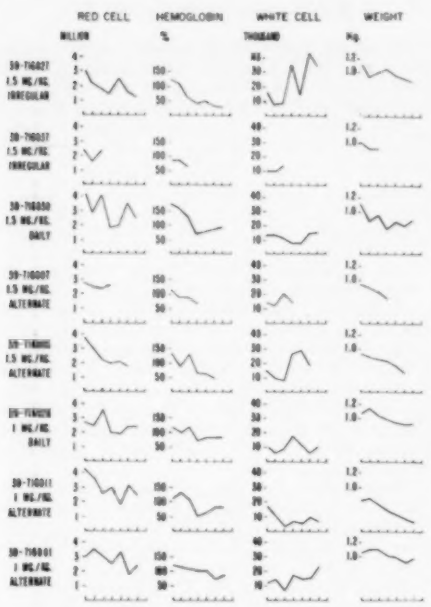
In this case, the differences in total phosphorus content of the tissues are significant in the case of chronic poisoning, and in the heart and liver in the case of acute poisoning. We may conclude, therefore, that a primary effect of phosphorus ingestion is a marked increase in the total phosphorus present in the heart tissues, and a marked decrease in the total phosphorus in the liver. The effect on the kidney is less marked, but it appears that the total phosphorus content is increased in cases of poisoning by this element.

Other factors studied were the effect of storage of the dead birds prior to removal of the tissues, and of the storage of the tissues after removal. No effects from either treatment could be noted.

Following the completion of the studies, four red-head ducks, supposedly killed by phosphorus

[3] *t*—a test of significance of difference. $t = \dfrac{M_1 - M_2}{\sqrt{\dfrac{\sigma_1^2}{N_1} + \dfrac{\sigma_2^2}{N_2}}}$

For a more complete explanation, see George Waddel Snedecor's "Statistical Methods," published by the Iowa State College Press in 1940.



The initial point of each graph represents the normal or pre-dosage reading. Other points are readings during dosing with variable time intervals as shown in Table 1.

Plate 1.--Blood and weight changes observed in ducks during experimental phosphorus poisoning

poisoning, were submitted by the Maryland Game Commission. Analysis of the tissues showed the total phosphorus contents to be: heart, 0.222%; liver, 0.124%; kidney, 0.168%.

Although it is possible that the differences between species might be sufficient to account for variation in these values, as contrasted with those found for the mallards and blacks, it appears probable that these birds also had been killed as the result of the ingestion of elemental phosphorus.

SUMMARY AND CONCLUSIONS

Black and mallard ducks were found to be highly susceptible to phosphorus poisoning, though marked individual variation in tolerance was shown. Three milligrams of white phosphorus per Kg. of body weight given in a single dose resulted in death of a black duck in six hours. One black drake survived ten daily dosings of 1 mg./-Kg. P and was active when sacrificed for tissues on the fourteenth day following the initial dose.

Histopathological tissue changes characteristic of phosphorus poisoning in mammals were found in both acute and chronic cases. Gross tissue changes observed in field cases were not duplicated in experimental birds. Difference in form and mode of intake of the phosphorus are believed to be the chief reasons for the variations shown. Gross lesions of duodenitis quite consistently present in field specimens were not found in experimental cases. Fatty degeneration of muscle tissues, liver, and kidneys was present in all cases of phosphorus poisoning. Pathologic changes, both gross and microscopic, indicated that all field specimens have been of the acute poisoning type. Some pathologic alterations of the blood of phosphorus-poisoned ducks are described and illustrated.

Seeming discrepancies between pathological findings and chemical analyses previously encountered in phosphorus-poisoned ducks have been satisfied.

Data have been presented showing that diagnosis by chemical analysis of stored tissues in cases of phosphorus poisoning can be accurately made.

REFERENCES

(1) Assoc. Official Agr. Chemists, "Official and Tentative Methods of Analysis," 6th ed., 1945.

(2) Stoloff, L. S., "Colorimetric Method for Phosphates," *Ind. Eng. Chem., Anal. Ed.*, **14**, 636(1942).

ARCHIV DER PHARMAZIE RESUMES PUBLICATION

After an interval of several years, *Archiv der Pharmazie und Berichte der deutschen pharmazeutischen Gesellschaft* resumted publication with the January, 1950, issue. This well-known scientific pharmaceutical journal will continue, as in the past, to publish reports of research in pharmaceutical chemistry, pharmacognosy, pharmacology, toxicology, galenical pharmacy, and related fields. The resumption of publication of *Archiv der Pharmazie* should be of especial interest to pharmacists in the United States who wish to keep informed on pharmaceutical developments in Germany.

The journal will be edited by Professor Friedrich von Bruchhausen, of Braunschweig, and it will be published by Verlag Chemie, G.m.b.H., Weinheim, a. d. Bergstrasse. More detailed information may be obtained from either of these sources.

Vitamin Stability in Oral Multivitamin Preparations*

By GEORGE B. STONE†

The stability of the more commonly used vitamins in vitamin concentrates and multivitamin preparations for oral administration has been studied in a variety of basic aqueous solvent mixtures of practical significance under normal storage conditions. The overage required for each vitamin under various conditions and in different formulations has been indicated in order to determine the excess of each vitamin necessary to maintain label declarations for normal storage periods.

One of the more apparent trends in the pharmaceutical industry with regard to vitamin products, which is receiving considerable attention by many manufacturers, is the preparation of liquid concentrates and multivitamin preparations for oral administration. These products are employed principally in pediatrics and geriatrics where the administration of vitamin concentrate and multivitamin tablets and capsules is not wholly satisfactory, and often impractical, in view of the difficulty frequently encountered in having small children or aged patients swallow pellets of any description.

Such preparations are liquid in character and water-miscible in order that they may be admixed with milk, fruit juices, etc., or added to semiliquid foods just before consumption. The usual carriers for these preparations consist of aqueous mixtures of propylene glycol, glycerin, ethanol, sucrose syrup, and similar solvents common to pharmaceutical elixirs and syrups. Sherry wine, honey, and corn syrup are sometimes found as constituents in the base formulation. These materials are employed for their preservative action in addition to increasing the viscosity and lending body to the product. The addition of the solvents to these preparations usually does not afford an increase in the solubility of the individual vitamins.

In the preparation of a liquid vitamin concentrate for oral administration the manufacturer usually attempts to attain as great a concentration of the vitamin in 0.6 cc. of solution as possible in order to obtain an effective therapeutic dosage. In the multivitamin preparation, the MDR (Minimum Daily Requirement) of the Federal Food and Drug Administration (1) is usually found in 0.6 cc. of solution.

For the most part the pure crystalline vitamins are employed as a source of the water-soluble vitamins in these preparations. However, it is not uncommon for rice bran extract, liver extract, and other natural vitamin concentrates to be used as sources. As regards the fat-soluble vitamins, A, D, and E, the usual natural concentrates or synthetic compounds are employed. In order to obtain a clear product these water-insoluble vitamins are incorporated by the use of certain dispersing or emulsifying agents.

Coloring agents are not generally used. The more common flavoring materials, when employed, are essential oil of orange and peppermint. If necessary these products are buffered to a *p*H range of 3.0 to 5.0 using any of the edible acids, but citric acid is commonly used when the flavoring agent is oil of orange.

In order to formulate a product of this nature it is necessary for the pharmacist or pharmaceutical chemist to have available rather specific knowledge of the stability and compatibility, as well as the solubility, of the vitamins in these preparations. Data concerning the solubility of most of the vitamins in water and in the usual pharmaceutical solvents, as well as in aqueous mixtures of the solvents, are available in standard reference works (2). The stability of the various vitamins in aqueous media has been rather widely investigated and reported in the published literature (3). However, the stability of the more important water-soluble vitamins in preparations of this nature has not been reported to any extent in the published literature and it is the object of this paper to relate the results of such an investigation.

EXPERIMENTAL

Propylene glycol and glycerin are the most common solvents employed for these preparations. A series of seven basic propylene glycol-containing solutions were prepared and are indicated in Table I. Employing the seven basic solutions, three individual sets of vitamin concentrates and two sets of multivitamin preparations were made up as indicated in Table II.

Sufficient weight of each vitamin was added to the solutions with stirring while heating the solution on the steam bath in the temperature range from 80–100° to yield a final volume of 500 ml. of solution. The vitamin C was added last after cooling to well below 60°. Since the object was to study the stability during storage, the vitamins were weighed

* Received May 24, 1949, from the Technical Service Department, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.
† The author is indebted to Miss Marjory Whitty and Miss Martha Karpus for carrying out many of the analytical determinations.

TABLE I.—BASIC SOLUTIONS PREPARED

Solution	% by Volume — Propylene Glycol N. F.	Glycerin U. S. P.	Simple Syrup U. S. P.	Distilled Water
A	100	..	..	..
B	75	25	..	..
C	50	50	..	..
D	75	..	25	..
E	50	..	50	..
F	75	..	..	25
G	50	..	..	50

TABLE II.—VITAMIN CONCENTRATE AND MULTIVITAMIN SOLUTIONS PREPARED

No.	Solution	Vitamin	Approximate Concentration, Mg./0.6 Cc.
1	B_1 Concentrate	Thiamine Hydrochloride U.S.P.	25
2	C Concentrate	Ascorbic Acid U.S.P.	100
3	B_1C Concentrate	Thiamine Hydrochloride U.S.P.	25
		Ascorbic Acid U.S.P.	50
4	B Complex	Thiamine Hydrochloride U.S.P.	1[a]
		Riboflavin, U.S.P.	2[a]
		Niacinamide, U.S.P.	4[b]
5	B Complex C	Thiamine Hydrochloride U.S.P.	1[a]
		Riboflavin U.S.P.	2[a]
		Niacinamide U.S.P.	4[b]
		Ascorbic Acid U.S.P.	30[a]

[a] Minimum Daily Requirement.
[b] Minimum Daily Requirement not yet established.

out approximately without attempting to obtain accurately the value indicated in Table II. After cooling, each solution was filtered brilliantly clear through a cotton pledget or filter paper and transferred to 8-ounce amber glass screw-topped bottles. These containers were stored at about 27° in diffused light for the periods indicated. At definite intervals the solutions were examined and assayed for vitamin potency. As time progressed the removal of samples increased the head space in the container as is encountered under practical conditions of storage and use. At the end of the study the final volume of solution amounted to about one-quarter of the original volume, leaving a head space equal to approximately three-quarters of the container volume. The samples removed were assayed for vitamin potency by standard chemical methods (2).

DISCUSSION

Thiamine Hydrochloride U. S. P.—Under the conditions of the experiment, thiamine hydrochloride U. S. P. appeared to be the least stable of vitamins investigated. The average percentage retention in the concentrate after a period of a year was about 83% with a range of 72–93% (Table III). In the concentrate with vitamin C the average retention for the same period was slightly higher, 84% and the range was the same (Table V). Retention values in the multivitamin preparation were much lower having an average value of 45% with a range of 38–54% (Tables VII and VIII). Higher retention values were evident in solutions containing the greater percentage of water and vice versa. The presence of ascorbic acid appeared to result in slightly higher retention values for the thiamine, but the increase was too little to be significant. Evidence, in the form of color development, probably as a result of the interaction of the vitamin with the sugar compounds, is to be noted from the results in Table VI.

TABLE III.—STABILITY OF THIAMINE HYDROCHLORIDE U. S. P. IN B_1 CONCENTRATE (SOLUTION 1)

Solution	0 Week — Mg./0.6 Cc.	Retention, %	13 Weeks[a] — Mg./0.6 Cc.	Retention, %	27 Weeks — Mg./0.6 Cc.	Retention, %	52 Weeks — Mg./0.6 Cc.	Retention, %
A	26.9	100	21.1	78.3	23.6	87.7	23.1	84.9
B	31.5	100	21.0	66.8	25.8	81.9	22.9	72.7
C	29.4	100	23.3	79.2	25.5	86.7	22.3	75.8
D	27.6	100	23.9	86.6	24.4	87.7	21.4	77.5
E	25.8	100	24.2	94.2	24.6	95.4	23.7	91.9
F	26.4	100	25.1	95.1	26.2	99.2	22.7	86.0
G	24.1	100	23.8	98.8	24.2	100	22.4	93.0

[a] Standard solution found to be incorrect. Values obviously too low.

TABLE IV.—STABILITY OF ASCORBIC ACID U. S. P. IN C CONCENTRATE (SOLUTION 2)

Solution	0 Weeks — Mg./0.6 Cc.	Retention, %	13 Weeks — Mg./0.6 Cc.	Retention, %	25 Weeks — Mg./0.6 Cc.	Retention, %	52 Weeks — Mg./0.6 Cc.	Retention, %
A	92.9	100	92.3	99.3	84.5	90.8[a]	76.2	81.8[a]
B	94.4	100	96.6	100	93.1	98.6	93.5	99.0
C	91.0	100	94.1	100	95.2	100	97.8	100
D	94.7	100	95.4	100	90.1	95.1	86.8	91.7
E	96.9	100	97.8	100	92.0	95.1	90.8	93.7
F	96.9	100	95.1	98.1	92.4	95.4	91.0	93.9
G	96.0	100	92.9	96.8	87.0	90.6	80.5	88.8

[a] Precipitation.

TABLE V.—STABILITY OF VITAMINS IN B_1 C CONCENTRATE (SOLUTION 3)

Solution	0 Week Mg./0.6 Cc.	0 Week Retention, %	13 Weeks Mg./0.6 Cc.	13 Weeks Retention, %	27 Weeks Mg./0.6 Cc.	27 Weeks Retention, %	52 Weeks Mg./0.6 Cc.	52 Weeks Retention, %
				Thiamine Hydrochloride U. S. P.				
A	25.6	100	24.1	94.1	24.2	94.5	24.0	93.2
B	26.7	100	25.6	95.9	22.2	83.1	20.9	72.5
C	25.2	100	23.5	93.3	23.8	94.5	23.3	90.7
D	27.0	100	24.2	89.6	25.2	93.3	22.0	77.0
E	28.4	100	25.6	90.1	24.7	87.0	23.4	78.4
F	28.4	100	25.9	91.2	25.8	90.0	24.1	82.1
G	25.8	100	24.7	95.8	25.2	97.7	24.0	93.0
				Ascorbic Acid U. S. P.				
A	46.1	100	45.4	98.5	44.8	97.2	43.3	93.9
B	47.1	100	47.8	100	47.1	100	45.4	96.2
C	47.4	100	47.6	100	48.5	100	47.1	99.4
D	48.0	100	47.3	98.5	46.5	96.9	43.4	91.5
E	48.7	100	48.9	100	47.7	98.0	44.6	92.5
F	49.5	100	48.2	97.4	46.0	92.9	43.9	88.6
G	47.8	100	46.4	97.1	43.0	90.0	38.5	80.6

TABLE VI.—COLOR DEVELOPMENT DURING STORAGE OF MULTIVITAMIN SOLUTIONS[a]

Solution	B_1 Concentrate, Solution 1	C Concentrate, Solution 2	B_1 C Concentrate, Solution 3
A	0	+++	+++++
B	+	+++	+++++
C	+	+++	+++++
D	++	++++	+++++
E	++	+++++	++++
F	0	+++++	++++
G	+	+++++	+++++

[a] After 52 weeks of storage at about 27°: 0, Colorless; +, very slight yellow tint; ++, light yellow; +++, dark yellow; ++++, orange-yellow; +++++, light orange; +++++, dark orange.

Riboflavin U. S. P.—The stability of the riboflavin in these preparations could not be ascertained because of its tendency to precipitate gradually from solution.

Niacinamide U. S. P.—This vitamin appears to be relatively stable in this type of preparation and stability values indicated an average retention of about 86% in the multivitamin preparations after a period of a year in storage, with a range from 75–97%. Retention values were significantly lower in the multivitamin concentrate containing ascorbic acid, an average reduction of 10% being noted (Tables VII and VIII).

Ascorbic Acid U. S. P.—Ascorbic acid is rather stable in preparations of this type. Tables IV and V indicate an average retention value after a year in the concentrate of about 92% with a range from 80–100%. In the multivitamin concentrate the retention values were lower and averaged 84% with a range from 79–88% (Table VIII). Conversely to the thiamine, formulations containing lesser amounts of water yield higher stability values for the ascorbic acid and vice versa. Discoloration by yellowing and darkening is an important consideration in concentrates containing this factor, and the degree of discoloration appears proportional to the percentage of water present (Table VI). Discoloration is also enhanced by the presence of thiamine. In the multivitamin preparations, due to the yellow color produced by the riboflavin, this discoloration problem can be eschewed.

No appreciable differences in the stability of the vitamins were noted in the solutions containing various solvents or proportions of solvent, except as previously noted.

TABLE VII.—STABILITY OF VITAMINS IN B COMPLEX SOLUTION (SOLUTION 4)

Solution	0 Week Mg./0.6 Cc.	0 Week Retention, %	13 Weeks Mg./0.6 Cc.	13 Weeks Retention, %	28 Weeks Mg./0.6 Cc.	28 Weeks Retention, %	54 Weeks Mg./0.6 Cc.	54 Weeks Retention, %
				Thiamine Hydrochloride, U. S. P.				
A	0.96	100	0.86	89.6	0.66	68.8	0.45	46.6
B	1.01	100	0.91	90.0	0.63	62.4	0.43	43.6
C	0.91	100	0.88	96.6	0.65	71.5	0.42	45.0
				Riboflavin U. S. P.[a]				
A	1.93	100	1.12	58.0	0.34	17.6	0.19	7.3
B	2.06	100	1.67	81.1	0.45	21.8	0.31	15.1
C	1.93	100	1.26	65.3	0.46	23.8	0.27	14.4
				Niacinamide, U. S. P.				
A	3.97	100	3.96	99.8	3.98	100	3.60	89.5
B	4.01	100	3.97	99.1	3.73	93.1	3.62	89.1
C	3.79	100	3.77	99.5	3.65	96.3	3.68	97.2

[a] Loss not attributed to destruction, but to precipitation.

TABLE VIII.—STABILITY OF VITAMINS IN B COMPLEX C SOLUTION (SOLUTION 5)

Solution	0 Week Mg./0.6 Cc.	0 Week Retention, %	13 Weeks Mg./0.6 Cc.	13 Weeks Retention, %	28 Weeks Mg./0.6 Cc.	28 Weeks Retention, %	54 Weeks Mg./0.6 Cc.	54 Weeks Retention, %
				Thiamine Hydrochloride U. S. P.				
A	0.89	100	0.84	94.4	0.63	70.8	0.34	38.2
B	0.89	100	0.86	96.5	0.62	69.6	0.38	42.7
C	0.88	100	0.83	94.4	0.62	70.5	0.48	54.4
				Riboflavin U. S. P.[a]				
A	2.00	100	1.50	75.0	0.32	16.0	0.24	12.0
B	1.99	100	1.10	55.3	0.49	24.6	0.30	15.3
C	2.07	100	1.70	82.2	0.52	25.1	0.33	16.2
				Niacinamide U. S. P.				
A	4.06	100			3.99	98.3	2.98	74.9
B	4.08	100			3.96	97.2	3.36	86.3
C	4.02	100			3.72	92.5	3.18	79.1
				Ascorbic Acid U. S. P.				
A	26.8	100	25.5	95.1	23.4	87.4	20.9	88.0
B	29.5	100	28.4	96.1	26.0	88.1	23.3	78.9
C	31.5	100	31.7	100	29.6	94.5	26.8	84.8

[a] Loss due to precipitation.

TABLE IX.—APPARENT OVERAGES OF VITAMINS REQUIRED IN LIQUID VITAMIN PREPARATIONS

	Excess Required, %—Concentrate	Excess Required, %—Multivitamin
Thiamine Hydrochloride U. S. P.	20–40[a]	50–100[a]
Niacinamide U. S. P.	10–20	10–20
Ascorbic Acid U. S. P.	15–25[a]	20–40[a]

[a] Increase or decrease to be determined by the proportion of water present in the formulation and period of storage.

CONCLUSIONS

1. The stability of the major water-soluble vitamins employed in the preparation of liquid vitamin concentrates and multivitamin products for oral administration has been studied under normal conditions of processing and storage.

2. The following percentage of excess of the vitamins appear to be required for formulations of this type in order to maintain label declarations for a period of one year under practical storage conditions.

REFERENCES

(1) Title 21, Code of Federal Regulations, Chapter 1, Sec. 125.3.
(2) "United States Pharmacopoeia," Thirteenth Revision, Mack Printing Co., Easton, Pa., 1947.
(3) United States Dispensatory, 24th Edition, J. B. Lippincott Company, Philadelphia, Pa., 1947.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Oxindole
Prodigiosin
Ulmic acid
Oxyhemoglobin
Andrenochrome
Hydrohydrastinine
L-Catechin
L-Perseitol
Myricetin
Curcumen, natural
Kaempferol
Quercetagin
Daidzin
Laccase
Physodic acid
Obtusatic acid
Coniferyl aldehyde
Hemipic acid

Production of Rutin from Buckwheat*,†

CHARLES F. KREWSON and JAMES F. COUCH‡

Methods for the extraction and purification of rutin from either green or dried buckwheat meal are described. The best solvent and extraction time for optimum yields of rutin were determined by comparing and evaluating various solvents. For the green plant ten minutes extraction time with hot 85 per cent isopropyl alcohol is recommended. For buckwheat meal 70 to 85 per cent isopropyl alcohol, either hot or cold, is the most efficient extraction solvent for rutin.

RUTIN IN quantity for clinical use was first produced from flue-cured tobacco in 1942. However, as mentioned in a previous publication (1) it had previously been identified in tobacco by other investigators. In our laboratory it was prepared by percolation with ethyl alcohol, a procedure similar to that used by Sando and Lloyd (10) for the extraction of elder flowers. The low yields from an expensive raw material led to a search for a more economical source of the glucoside. This resulted in the selection of buckwheat (5) as the most promising of the various plants investigated. As previously mentioned (5) rutin was first isolated from buckwheat by Schunck in 1860. As a matter of historical record, rutin from buckwheat was first prepared in quantity from the fresh green plant in June, 1944, by a technique similar to that used for flue-cured tobacco. Several articles have since been published on production of rutin from buckwheat (3, 4, 8) for the purpose of giving information to manufacturers. The purpose of this paper is to describe the methods used for solvent extraction of buckwheat and to present some of the data on which manufacturing recommendations have been based. The extraction procedures have been changed from time to time as more knowledge of the chemical and physical properties of rutin became available.

At present, the most widely used method for preparing rutin employs hot isopropyl alcohol extraction of either fresh green buckwheat or a leaf meal obtained by drying the green plant. Optimum conditions for production of the leaf meal have been described (8).

Tartary buckwheat has been used for most of the experiments reported here because data obtained over three growing seasons (6) have demonstrated the superiority of this variety over other common varieties.

* Received October 27, 1949 from the Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia 18, Pa.

† Presented in part at the Meeting-in-Miniature of the Philadelphia Section of the American Chemical Society, January 20, 1949.

‡ The authors thank J. Naghski for timely suggestions; R. W. Bruch for technical assistance; J. Naghski and Charles Fenske for rutin analyses; and W. L. Porter and M. L. Swain for spectrophotometric data.

EXPERIMENTAL

Four methods for production of rutin will be presented in this paper. They are (*a*) cold solvent extraction of green Tartary buckwheat, (*b*) cold solvent extraction of commercial Japanese buckwheat whole meal, (*c*) hot solvent extraction of green Tartary buckwheat, and (*d*) hot solvent extraction of Tartary buckwheat leaf meal.

Because of many variables involved in the extraction and the pressing need for an adequate supply of rutin for clinical investigations, few significant data on factors influencing yield of rutin were obtained from the first fourteen preparations, in which from 50 to 500 pounds of fresh plant were processed. Results obtained from these experiments together with solubility data (11) indicated, however, that the strength of the solvent was an important factor influencing yield. The experiments under (*a*) were made to evaluate the efficacy of different strengths of ethyl alcohol in extracting rutin from the green plant.

Cold Solvent Extraction of Green Tartary Buckwheat

Procedure.—Into a 100-gallon stainless steel tank were placed 100 pounds of freshly harvested green buckwheat plant (minus roots). Previous experiments indicated that cutting or chopping the plant does not give more efficient extraction; it serves only to facilitate packing the plant into a tank. A 1-inch layer of absorbent cotton covered with a 4-inch layer of excelsior placed over the valve of the tank previous to addition of the buckwheat served to strain off the dirt. The buckwheat was covered with 50 gallons of ethyl alcohol (75% by volume), which was circulated at the rate of about 6 gallons per minute for four hours. The buckwheat was then allowed to macerate overnight. The first extract (45.6 gallons) was pumped to the evaporator through a small stainless steel filter press equipped with 1.5 square feet of heavy filter sheets (Republic Seitz Sheet No. K-5 was used[1]) backed with canvas. The marc was covered with 50 gallons of ethyl alcohol (75% by volume), and the process used in the first extraction was repeated.

The first extract was evaporated at atmospheric pressure to 12 gallons and held overnight before the addition of the second extract and washings. After twenty hours the second extract (47.6 gallons) was withdrawn and filtered. The marc was washed twice with 10 gallons of 75% ethyl alcohol; the alcohol was circulated for fifteen minutes each

[1] The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

time. These washings and an additional 2 gallons used to wash out the filter press were pumped to the evaporator with the second extract.

The combined extracts and washings were evaporated to 6 gallons. The concentrate was strained through glass wool (2–4) to remove fats[2] and pumped into a stainless steel, steam-heated holding tank containing 6 gallons of boiling water. After boiling for ten minutes to facilitate agglomeration of fats, the concentrate was filtered through 4 square feet of heavy filter sheets (K-5) backed with canvas, after which the filter press was washed with 2 gallons of boiling water.

The filtered solution was allowed to stand in a stainless steel tank overnight for rutin to crystallize. The rutin was then filtered off on heavy canvas, slurried in a small quantity of cold water, and refiltered. The slurrying was repeated. The crude rutin was dried at 110° to constant weight. Analysis of the air-dried marc showed no rutin.

This experiment was repeated with menstrua of different alcoholic strengths. Table I contains a summary of the data obtained.

Discussion.—In general, yields of crude rutin, were low when cold alcohol was used. The reason is not definitely known, but it may be due to the failure of cold alcohol to penetrate the cell wall of the plant in sufficient quantity to inhibit enzymatic destruction of rutin. The data on ethyl alcohol in the extracts (Table I) lends some support to this idea. From the alcohol strength of the extracts, it is evident that the solvent does not reach equilibrium with the water in the plant cells in the extraction period of twenty hours. The enzyme system of the buckwheat plant is now under investigation in this laboratory.

The best yield of rutin was obtained when cold alcohol of 75% strength by volume was used. This is in close agreement with solubility data for rutin-alcohol-water mixtures. Previous experiments, as well as subsequent experiments showed the impracticability of extracting buckwheat with solvent strength as low as 65% by volume. This was due to formation of fat emulsions on evaporation of the extracts, which seriously interfered with precipitation of rutin.

The data indicate also a slight gain in yield of rutin with separate working-up of each extract as obtained. This is shown by comparison of Experiment 166-164 with 166-148. A slight superiority of cold isopropyl alcohol over cold ethyl alcohol as an extractant is indicated by comparison of Experiment 166-171 with 166-148. After suitable methods for the preparation of a leaf meal with good rutin content had been developed, (8), studies were made on extraction procedures for buckwheat meal. These together with those on extraction of the green plant led to the selection of isopropyl alcohol for use in further studies. In addition to being more efficient with respect to yield of rutin especially when used in hot extraction, it has the added advantage of lower cost compared to the denatured alcohols and it is less expensive because of its lower heat of vaporization, which results in power conservation. Moreover, isopropyl alcohol is free of Internal Revenue restrictions.

Cold-Solvent Extraction of Commercial Japanese Buckwheat Whole Meal

In previous cold-solvent extractions of buckwheat meal, approximately 70% alcohol was indicated by solubility data as the most promising solvent. Typical of the percolation data are those of Experiment 166-18 (Tables II and III), in which twenty-hour extracts were made until the marc was free of rutin. In this experiment two 100-gallon stainless steel tanks were packed with buckwheat meal. The meal in the first tank (193.5 pounds or 87.8 Kg.) was covered with 80 gallons of 70% ethyl alcohol and allowed to macerate overnight. The first extract of 49 gallons was withdrawn and worked up for crude rutin. New solvent was added to cover the marc, which was again allowed to stand overnight. This extract was withdrawn and used, together with sufficient additional 70% alcohol to make 80 gallons, to cover the 175 pounds (79.4 Kg.) of meal in the second tank. Fresh solvent was again used to cover the marc in the first tank. After overnight maceration the extract was withdrawn

[2] The chemical composition of these fats, a by-product in the rutin manufacturing industry, will be the subject of another paper.

TABLE I.—EFFICIENCY OF ETHYL ALCOHOL AT VARIOUS STRENGTHS IN COLD-SOLVENT EXTRACTION OF GREEN TARTARY BUCKWHEAT[a]

Experiment No.	Ethyl Alcohol Strength (by Vol.), %	Ethyl Alcohol in Extracts, %		Analysis of Plant, %		Rutin in 45.4 Kg. of Plant (Based on Crude Rutin Analytical Value), Gm.	Yield of Crude Rutin Based on Analytical Value	
		Extr. 1	Extr. 2	Moisture	Crude Rutin, M.F.B.		Gm.	%
166-128	75	65	74	85.5	2.97	199.4	120.9	60.6
166-134	80	73	78	83.6	2.58	191.9	103.9	54.1
166-140	85	81	85	88.5	2.88	192.1	94.1	48.9
166-142	90	80	88	84.8	2.97	204.6	89.4	43.7
166-148	95	90	95	90.5	3.87	166.7	86.3	51.8
166-164[b]	95	..	...	91.3	3.84	151.6	82.9	54.7
166-144	100	90	100	85.9	2.68	171.4	87.5	51.1
166-150	100[c]	90	100	82.9	3.39	269.0	140.0	52.0
166-171	98–99[c]	..	...	87.9	3.55	194.8	105.9	54.4

[a] 45.4 Kg., 100 lb., was extracted twice for twenty hours each with 50-gallon portions of solvent followed by two 10-gallon washes.
[b] Extracts were worked up separately for crude rutin.
[c] Isopropyl alcohol.

TABLE II.—EXPERIMENT 166-18: EXTRACTION OF RUTIN FROM JAPANESE BUCKWHEAT MEAL BY PERCOLATION WITH 70% ETHYL ALCOHOL

Extract No.	Ethanol Used to Cover, Gal.	Ethanol Withdrawn, Gal.	Extract Concentrated to, Gal.	Yield of Crude Rutin, Gm.	Portion of Total Crude Rutin, %
1	80	49	4	1100.0	20.4
2	80	28	6	947.0	17.5
3	50	29	5	830.0	15.4
4	30	46	4	915.0	17.0
5	17	17	4	355.0	6.6
6	32	32	5	267.0	4.9
7	34	34	5	325.0	6.0
8	32	32	4	250.0	4.6
9	35	35	3	188.7	3.5
10	25	25	2	105.0	1.9
11	37	37	2	69.0	1.3
12	37	37	2	41.0	0.8
13	30	30	2	2.5	> 0.1
14	40	40			
	Total 559	471		5395.2	

TABLE III.—EXTRACTION EFFICIENCY OF VARIOUS SOLVENTS IN COLD SOLVENT EXTRACTION OF JAPANESE BUCKWHEAT MEAL

Experiment No.	Solvent in Extraction	Total Quantity of Solvent, Gal.	Weight of Meal, Kg.	Analysis of Meal, %		Rutin in Meal (Based on Crude Rutin Anal. Value), Gm.	Yield of Crude Rutin Based on Crude Rutin, Analytical Value		Yield of Refined Rutin, Based on Crude Rutin Analytical Value	
				Moisture	Crude Rutin M.F.B.		Gm.	%	Gm.	%
166-18[a]	70% ethyl alcohol	559	167.2	9.00	2.61	3970.1	5395.2	135.9	2955.2	74.4
166-42[b]	70% ethyl alcohol	255[b]	45.7	6.53	2.47	1055.8	1210.0	114.6	802.4	76.0
166-46[c]	70% ethyl alcohol	270[c]	39.9	6.85	2.69	999.4	1114.1	111.5	764.4	76.5
166-55[d]	70% ethyl alcohol	40[e]	4.54	6.53	2.66	112.8	120.3[f]	106.6	97.9	86.9
166-57[d]	70% isopropyl alcohol	40[e]	4.54	6.53	2.66	112.8	116.7	103.5	99.4	88.1
166-59[d]	70% methyl alcohol	40[e]	4.54	6.53	2.66	112.8	105.6	93.6	86.8	77.0
166-82[d]	70% acetone	0.7[g]	0.11	8.60	3.08	3.1	3.4	109.7	2.52	81.8

[a] Rutin was refined by recrystallization from boiling water (3, 5), pharmaceutical quality.
[b] Only 4.0 Gm. rutin was extracted by an additional 70-gallon, twenty-hour extraction.
[c] No rutin obtained by an additional 70-gallon, twenty-hour extraction.
[d] Rutin refined by recrystallization from 10% isopropyl alcohol (3, 5), not pharmaceutical quality.
[e] One 30-gallon, twenty-hour extraction plus two 5-gallon washings. No rutin was found in marc.
[f] Only 2.6 Gm. of additional rutin was obtained by another 10-gallon, twenty-hour extraction.
[g] One 0.7-gallon, twenty-hour extraction plus two 0.2-gallon washings. No rutin was found in marc.

from the second tank to be worked up for crude rutin. This process was repeated until an extract gave no appreciable rutin on evaporation. Table II shows the crude rutin yield by extracts. Crude and refined yields are recorded in Table III for comparative purposes. The comparative data show that all the available rutin in the meal is extracted by this percolation technique. This procedure offers a convenient method when countercurrent extraction is desirable, and when either quantity of solvent available and (or) size of evaporator are limiting factors in production of rutin from buckwheat meal. Extracts may be withdrawn from percolators in convenient quantities and worked up for crude rutin at suitable intervals.

With a higher solvent-to-plant ratio, it was possible to recover the rutin with fewer extractions. The following procedure was used when only three extractions were made (Experiments 166-42 and 166-46, Table III).

Procedure.—Into a 100-gallon stainless steel tank[3] was placed a false bottom consisting of a 4- to 6-inch layer of excelsior, over which a layer of cheesecloth was spread and tucked in around the edges. The false bottom was anchored with dry meal before addition of solvent. Buckwheat meal (total, 39.9 Kg.) and 70% ethyl alcohol (115 gallons) were then added until the tank was filled. The solvent was continuously recirculated from the bottom to top of the tank at the rate of about 1 gallon per minute for twenty hours. This period was longer than necessary but was used as a convenient day-to-day working period. This action pumps the unscreened fines to the top of the tank and makes a filter bed of the meal, so that the extract soon becomes clear and filtration of the extract before it is transferred to the evaporator is unnecessary. After twenty hours the extract (approximately 85 gallons) was drawn off and evaporated to 8 to 10 gallons at atmospheric pressure. The boiling concentrate was strained through glass wool and then filtered through heavy paper sheets (K-5). The filtrate and washings were allowed to stand for twenty hours for rutin to crystallize. The crude product was filtered off on heavy canvas, slurried with cold water, refiltered, and washed with small portions of cold water. The dry weight of the

[3] The tank used is rated at 100-gallon capacity but actually holds 120 gallons.

TABLE IV.—EXTRACTION EFFICIENCY OF VARIOUS STRENGTHS OF SOLVENT AND EFFECT OF BOILING TIME ON YIELD OF RUTIN IN HOT ISOPROPYL ALCOHOL EXTRACTION OF GREEN TARTARY BUCKWHEAT[a]

Experiment No.	Solvent Strength (by Vol.), %	Analysis of Plant, % Corrected Rutin, M. F. B.		Rutin in 22.7 Kg. (Based on Corrected Rutin Analytical Value), Gm.	Yield of Corrected Rutin Gm.	%
		Moisture				
194-121	65	83.3	4.12	156.0	102.5	65.7
194-120	75	87.2	5.40	156.8	119.9	76.5
166-173	85	88.8	3.93	99.8	81.7	81.8
166-175	85	88.2	3.58	95.8	79.7	83.2
166-185	85	86.0	4.16	132.1	108.5	82.1
194-124[b]	85	88.9	5.00	125.9	94.9	75.4
194-122[c]	85	89.9	5.19	118.9	85.5	71.9
166-192	95	82.4	3.06	122.2	84.6	69.2
166-190	98–99	82.4	3.09	123.3	79.7	64.6

[a] 22.7 Kg. (50 pounds) green buckwheat was extracted 3 times for ten-minute periods with 20, 12, and 12 gallons, respectively, of solvent.
[b] First extraction period was sixty minutes.
[c] First extraction period was one hundred and twenty minutes.

TABLE V.—EFFICIENCY OF VARIOUS SOLVENTS IN HOT-SOLVENT EXTRACTION OF GREEN TARTARY BUCKWHEAT[a]

Experiment No.	Solvent	Analysis of Plant, % Corrected Rutin, M. F. B.		Rutin in 22.7 Kg. (Based on Corrected Rutin Analytical Value), Gm.	Yield of Corrected Rutin Gm.	%
		Moisture				
166-175	85% isopropyl alcohol	88.2	3.58	95.8	79.7	83.2
194-128	85% ethyl alcohol	84.1	3.85	138.8	105.0	75.6
194-125	85% methyl alcohol	86.4	4.54	140.0	83.7	59.8
194-129	75% methyl alcohol	86.2	4.76	149.0	73.0	49.0[b]
194-130[c]	85% acetone	83.0	3.97	3.37	3.10	92.0

[a] 22.7 Kg. (50 pounds) green plant was extracted three times for ten-minute periods, with 20, 12, and 12 gallons, respectively, of boiling solvent.
[b] Analysis of marc showed 21.7% additional rutin.
[c] One-half Kg. sample was used.

crude rutin obtained was 795.0 Gm. or 71.4% of the total crude rutin obtained from all extracts (Experiment 166-46, Table III). The marc was extracted two more times for twenty hours each with 70 gallons of 70% ethyl alcohol. Each of these extracts was evaporated to approximately 6 gallons and handled in the same way as the first extract. From the second extract was obtained 216.0 Gm. or 19.4% of the total weight of crude rutin; from the third extract, 103.1 Gm. or 9.2% by weight of the total crude rutin. A fourth extract yielded no rutin.

Discussion.—In Experiments 166-55, 166-57, 166-59, and 166-82 of Table III, which illustrate the use of still higher solvent-to-meal ratios, good results were obtained by one extraction followed by several washings to remove entrained solvent-bearing rutin.

Attempts to extract buckwheat meal with strong cold acetone have been unsuccessful, and this experience, at an early date in the rutin program, discouraged further work with this solvent. However, if the meal were first extracted with a fat solvent, such as benzene, the rutin might be extracted with strong acetone. Extraction with 70% acetone on a small scale (Experiment 166-82, Table III) gave a satisfactory yield. Further investigation with this solvent is in progress.

No corrected rutin yields are given here because the data were obtained before the variability of benzene soluble and alcohol-insoluble impurities in crude rutin was realized. However, data on the yield of "refined" rutin are included. The values for Experiments 166-18, 166-42, and 166-46 were obtained by purifying the manufactured rutin by crystallization from boiling water, which produced rutin of pharmaceutical quality; those for Experiments 166-55, 166-57, 166-59, and 166-82 were obtained by crystallization from 10% isopropyl alcohol (rutin not of pharmaceutical quality).

Hot-Solvent Extraction of Green Tartary Buckwheat

Several preliminary experiments were made on hot extraction of green buckwheat with strong ethyl alcohol (95% by volume). In one, 50 pounds of green plant were extracted with 20, 12, and 12 gallons[4] of boiling alcohol for ten-minute periods. The yield of crude rutin was 79.4%, as compared with 54.7% for cold alcohol (Experiment 166-164, Table I). When the boiling periods were increased to sixty minutes each, the yield was 62.3%. Use of 85% isopropanol for thirty-minute boiling periods gave a crude rutin yield of 69.3%. These preliminary tests indicated that hot extraction gives a higher yield than cold extraction and that possibly prolonged contact of hot solvent with green buckwheat may result in lower yields.

Table IV summarizes the data of experiments planned to determine the optimum solvent strength

[4] A fourth extraction was not necessary because only 0.7 Gm. of rutin was obtained in an additional 12-gallon extraction.

and heating time for extraction of green Tartary buckwheat with isopropyl alcohol. Table V summarizes the data on the efficiency of several of the most promising solvents.

At an early date evidence indicated that crude rutin obtained both in the manufacturing process and by analysis[5] contained two main types of impurities in different amounts—"benzene solubles" and "alcohol insolubles." In these experiments the benzene solubles and alcohol insolubles in the manufactured rutin ranged from 0.05–3.33% and 1.22–7.79%, respectively, whereas in the analytical control samples the range was 0.81–6.33% and 3.58–13.7%, respectively. Since these impurities showed such wide variations, it became essential to correct all rutin values before an evaluation could be made of the factors affecting yields. Rutin freed of these impurities is hereafter referred to as "corrected."

The following extraction procedure is typical of that used in the experiments presented in Tables IV and V.

Procedure.—To a 50-gallon stainless steel, steam-heated kettle were added 50 pounds of whole freshly harvested buckwheat (minus roots). Sufficient isopropyl alcohol (85% by volume) was added to cover the plant (20 gallons). The alcohol was brought to a boil as rapidly as possible (six minutes) and held at boiling temperature for ten minutes. The first hot extract was drawn off, filtered by pressure through 2 square feet of heavy filter sheets (K-5)backed with canvas, and pumped into an evaporator. Similarly, a second and a third ten-minute extract were made; 12 gallons of 85% isopropyl alcohol was enough to cover the marc in each case. Each extract was worked up separately for crude rutin.

The first extract, approximately 16 gallons, was evaporated at atmospheric pressure to 2.5 to 3 gallons. The boiling concentrate was strained through glass wool and filtered through about 1 square foot of heavy filter sheet. The evaporator and filter were washed with 0.5 gallon of boiling water, and the filtrate and washings were immediately cooled by the addition of 10 to 15 pounds of crushed ice until the temperature was about 5°. After cooling, the mixture containing crude crystallized rutin was allowed to stand at low temperature for one hour, after which it was filtered off on hard-surfaced paper, thoroughly washed with small portions of cold water, and dried at 110° to the constant weight of 64.5 Gm. No further precipitation of rutin occurred after the mother liquors had stood for forty-eight hour periods in a cold room (temperature about 5°). The second and third extracts of approximately 12 gallons each were evaporated to about 0.75 gallon, strained, filtered, and cooled with about 5 pounds of crushed ice. These solutions were allowed to stand for one hour for complete crystallization of crude rutin. The dried crude rutin from the second extract weighed 12.0 Gm., and that from the third extract, 6.5 Gm. Previous experience had shown that a fourth extraction was unnecessary. The entire experiment, from the cutting of the green buckwheat, was completed in a single eight-hour working day.

Discussion.—The data in Table IV show that 85% isopropyl alcohol and a ten-minute boiling extraction period gave the maximum yield of rutin. Heating for longer periods, as in Experiments 194-124 and 194-122, lowered the yield of rutin. An extraction with 55% alcohol (not shown in the table) was highly unsatisfactory because the fats emulsified when the extracts were evaporated. Large amounts of salt had to be used to agglomerate the fat, and then completeness of crystallization was not certain even after forty-eight hours at low temperature. With low alcohol strength, evaporation of solvent was also slow and impracticable, especially at atmospheric pressure.

A breakdown of yields of crude rutin in Experiment 166-175, which is representative of those obtained in other experiments with hot-solvent extraction of green buckwheat, showed that 77.7, 14.5 and 7.8% of the total weight of crude rutin was present in the first, second, and third extract, respectively. It would appear that the last two extractions serve only to wash out rutin-bearing solution from the marc. It seems reasonably safe to assume that complete extraction occurs with one ten-minute hot extraction. It has been suggested that in commercial operations, if batch extraction methods are used, possibly no more than two extractions are needed, one of which might be a thorough hot washing. When a third extraction appears economically feasible, this extract could be used for the first extraction of a new batch.

Data in Table V indicate the superiority of 85% isopropyl alcohol over ethyl and methyl alcohols for the extraction of green buckwheat. The possibilities of dilute acetone have not been thoroughly investigated, but in a preliminary experiment in which a 0.5-Kg. sample was used (Experiment 194-130) 85% hot acetone appeared promising as a solvent for green buckwheat.

Hot-Solvent Extraction of Tartary Buckwheat Leaf Meal

Table VI shows the data for the optimum conditions with regard to the strength of hot solvent and length of heating time for extraction of buckwheat meal. In a preliminary experiment (not included in the table) in which hot 70% methyl alcohol was used as a solvent, the corrected yield of rutin was 60.7%, only about two-thirds that obtained with isopropyl alcohol under similar conditions. The technique used in Experiment 194-9 which is typical of that recommended for the extraction of leaf meal, was as follows.

Procedure.—A false bottom was placed in a 50-gallon stainless steel, steam-heated kettle, and 5 pounds of meal were put in to serve as an anchor. Fifteen gallons of 75% isopropyl alcohol were added, followed by an additional 5 pounds of meal. The alcohol required seven minutes to reach boiling temperature, and the mixture was kept at this point for ten minutes. The first extract was pumped into the evaporator through 2 square feet of heavy paper sheets (K-5) backed with canvas. One gallon of 75% isopropyl alcohol was used to wash the filter press free of the first extract solution. The 12 gallons (approximate) of extract obtained were evaporated at atmospheric pressure to 2 gallons. Three

[5] The method used in these analyses, together with additional data showing the need for correction of the yield of crude rutin has been published since the preparation of this manuscript. Naghski, J., Fenske, C. S., Jr., Krewson, C. F., and Couch, J. F., "Determination of Rutin in Plant Materials," *U. S. Dept. Agr., Bur. Agr. and Ind. Chem.*, AIC-236 (Eastern Regional Research Laboratory), August, 1949. (Processed.)

TABLE VI.—EXTRACTION EFFICIENCY OF VARIOUS STRENGTHS OF SOLVENT AND EFFECT OF BOILING TIME ON YIELD OF RUTIN IN HOT ISOPROPYL ALCOHOL EXTRACTION OF TARTARY BUCKWHEAT LEAF MEAL[a]

Experiment No.	Solvent Strength (by Vol.), %	Yield of Crude Rutin, Based on Crude Rutin Analytical Value Gm.	%	Benzene Solubles in Crude Rutin, %	Alcohol Insolubles in Crude Rutin, %	Corrected Yield of Rutin Gm.	%	Crude Rutin in Marc, by Analysis Gm.	%
194-19	55	185.3	97.6	16.92	12.03	131.7	74.3	3.18	1.79
194-17	65	183.5	96.7	0.51	8.49	167.0	94.2	2.84	1.60
194-29	70	187.0	98.5	0.13	6.72	174.2	98.3	1.65	0.93
194-5[b]	75	180.1	94.9	0.09	5.46	170.1	95.9	5.52	3.11
194-9	75	185.4	97.7	0.09	5.19	175.6	99.0	6.90[c]	3.90
194-13[d]	75	177.0	93.3	0.06	4.63	168.7	95.2	None found	None found
194-15	85	175.8	92.6	0.09	0.98	175.7	99.1	8.70	4.91
194-27	90	132.3	69.7	0.08	0.45	131.6	74.2	43.9	24.8
194-25	98–99	41.9	22.1	0.29	3.04	40.5	22.8	137.2	77.4

[a] 4.54 Kg. (10 pounds) of meal was extracted three times for ten-minute periods with 15, 6, and 6 gallons, respectively, of boiling solvent. Meal analyzed 8.10% moisture, 4.55% crude rutin. Rutin contained 5.16% benzene solubles, 0.36% alcohol insolubles. Corrected yield of rutin is 4.30%.

[b] Ten gallons of solvent instead of 15 gallons was used for first extract.

[c] Obtained by two additional 6-gallon extractions.

[d] First extraction period was one hundred and twenty minutes.

gallons of boiling water were added, and boiling was continued for fifteen minutes. An additional gallon of boiling water was added, and the boiling concentrate was strained through glass wool and filtered through heavy filter sheets; about 2 square feet of filtering area were used. The equipment was washed out with a small quantity of boiling water. To the hot filtrate and washings (about 5½ gallons) were added 20 pounds of crushed ice. After one hour, the crude rutin was filtered off on hard-surfaced paper, washed three times with 0.25-gallon portions of cold water and dried at 110° to a constant weight of 138.0 Gm.

A second extract was made with 6 gallons of 75% isopropyl alcohol (by boiling for ten minutes) immediately after removal of the first extract. This second extract was evaporated to 1 gallon, diluted with 2 gallons of boiling water, boiled for fifteen minutes, strained, and filtered. To the rutin filtrate (approximately 3 gallons) were added 10 pounds of crushed ice for rapid cooling. After one hour the rutin was filtered off, washed and dried at 110° to a constant weight of 37.0 Gm.

A third extract was made by the same technique as that described for the second extract. However, since this extract contained less rutin, it was evaporated to 0.5 gallon instead of 1 gallon, in order to insure complete crystallization of rutin in one hour. The yield of crude rutin from the third extract was 10.4 Gm., making the total yield from the three extracts 185.4 Gm. Since a total yield of only 5.9 Gm. was obtained from two additional 6-gallon extracts, just three extracts were made in all subsequent experiments. Data for these experiments are included in Table VI. The entire procedure—making the three extractions and placing the crude rutin in the oven to dry—required one working day.

Discussion.—Again the necessity for correcting the figure for yield of crude rutin of both manufactured and analytically determined rutin for benzene solubles and alcohol insolubles is demonstrated by the data in Table VI. Reasonable checks can be obtained in the extraction of buckwheat meal with hot solvent as demonstrated in the extraction of green plant with hot solvent, provided corrected rutin values are compared.

From the quantity of benzene solubles and alcohol insolubles in the crude rutin, together with corrected yield figures in Table VI, it appears that 85% is the optimum strength to be recommended, although any concentration from 70% to 85% is practical. With alcohol of lower strength, fat emulsion difficulties are encountered in evaporation of solvent, and large amounts of electrolyte must be added to break such emulsions. Even so, it will be noticed from Experiments 194-19 and 194-17 that with 55% and 65% isopropyl alcohol extraction the benzene solubles and alcohol insolubles in the crude rutin are increased with decrease in solvent strength. This means that greater difficulty will be encountered in refining to a pharmaceutically acceptable product. Above 85% strength, the yield of rutin sharply diminishes, which is in close agreement with solubility data for rutin in boiling isopropyl alcohol-water mixtures. In extraction of buckwheat meal, heating for

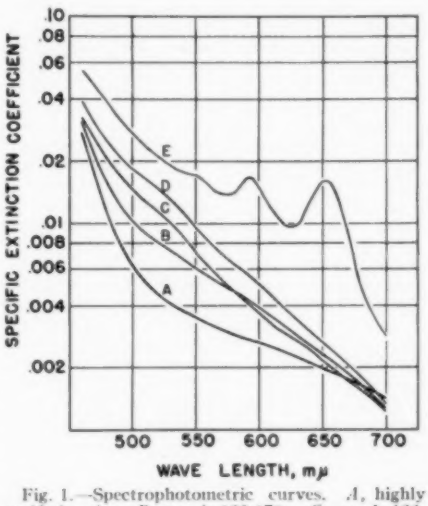


Fig. 1.—Spectrophotometric curves. *A*, highly purified rutin; *B*, sample 166-179; *C*, sample 166-46; *D*, sample 194-9; *E*, crude rutin. Curves for other samples in Table VII fall within limits bounded by *B*, *C*, and *D*.

TABLE VII.—PURITY OF SAMPLES OF RUTIN PREPARED BY VARIOUS METHODS OF EXTRACTION[a]

Experiment No.	Crude Rutin Prepared by—	Rutin after Refining, Gm.	Rutin,[b] %	Quercetin,[b] %
166-175	Hot 85% isopropyl alcohol extraction of green buckwheat	7.66	98.5	0.8
166-179	Hot 75% isopropyl alcohol extraction of green buckwheat	7.32	97.4	0.2
166-140	Cold 85% ethyl alcohol extraction of green buckwheat	7.73	99.2	1.6
166-128	Cold 75% ethyl alcohol extraction of green buckwheat	7.33	98.4	1.6
194-15	Hot 85% isopropyl alcohol extraction of leaf meal	7.83	97.8	1.0
194-9	Hot 75% isopropyl alcohol extraction of leaf meal	7.33	97.4	1.4
166-46	Cold 70% ethyl alcohol extraction of whole meal	764.5[c]	96.1	2.2

[a] Purified by recrystallization of 10.0 Gm. of crude rutin from boiling water; 5.0 Gm. of silica gel was used for removal of red pigment. Not more than a trace of absolute ethanol insoluble impurities was present.
[b] Rutin, quercetin, red pigment, and chlorophyll were determined by the method of Porter, Brice, Copley, and Couch (9). Red pigment in each sample was less than 0.0001%; chlorophyll was less than 0.001%.
[c] From recrystallization of 1114 Gm. of crude rutin.

more than ten minutes was not so deleterious to rutin yields as it was in extraction of green buckwheat. However, there is a definite loss from prolonged heating; heating a concentrated extract for twelve hours resulted in a 14% loss of crude rutin.

REFINING CRUDE RUTIN

Instructions for refining crude rutin have been presented in some detail elsewhere (3, 4). Table VII gives representative data illustrating the purification of rutin to pharmaceutical quality. Samples of crude rutin prepared by the various methods of extraction were purified. The data in Table VII and the spectrophotometric curves in Fig. 1 (9) show that there was little if any difference in the quality of the various crude rutin samples, inasmuch as they all yielded rutin of comparable quality when refined by the same process. The refining procedure may be outlined as follows:

A sample of dry rutin (dried to constant weight at 110°), weighing 10.0 Gm., was dissolved in 2 L. of boiling water. The boiling solution was filtered through a heavy filter sheet (K-5), and the filtrate was reheated to boiling. To the boiling solution were added, with continuous stirring, 5.0 Gm. of silica gel (28–200 mesh), and the boiling was continued for five minutes. The solution was filtered through a heavy filter sheet (S-3 of Republic Seitz Corp.) backed with canvas. The rutin solution was cooled and held at a low temperature (5° to 10°) for one hour. The refined rutin was then filtered on hard-surface paper (preferably rayon) and dried to constant weight at 110°. This technique is equally applicable to the purification of large quantities of rutin, as shown in Experiment 166-46 of Table VII.

The quantity of rutin which could be recovered from the mother liquor after recrystallization of the crude product from hot water was determined in Experiment 166-46. The 45 gallons were concentrated to about 2 to 3 gallons and cooled, and the *p*H was adjusted with sulfuric acid to 2.5. The cold solution was allowed to stand for two hours, and the crude rutin was filtered off. The dry weight of this crude material was 22.0 Gm. After two recrystallizations, required to refine it to pharmaceutical quality, 8.4 Gm. of rutin were obtained.

SUMMARY

Hot and cold organic-solvent methods for production of rutin from either green buckwheat plant or dried buckwheat meal are described. The efficiency of various solvents was evaluated, and the best solvent strength and extraction time for optimum yields of rutin were determined. Extraction for ten minutes with hot 85% isopropyl alcohol is recommended for the green plant, since cold solvents give lower yields. Buckwheat meal may be extracted efficiently with either hot or cold solvent, although the hot solvent materially reduces the processing time; 70% to 85% isopropyl alcohol is preferred.

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Interaction of *para*-Aminosalicylic Acid and Streptomycin on *Escherichia Coli**,†

By KSHITISH J. DIVATIA, JEAN DUFRENOY, and ROBERTSON PRATT

The antibacterial action of streptomycin *in vitro* against *Escherichia coli* is depressed or enhanced in the presence of low concentrations of NaPAS, depending on the concentration of the latter, in the nutrient substrate. No effect of NaPAS on the action of streptomycin on *E. coli* was observed when both drugs were permitted to diffuse together from a central reservoir on assay plates.

PARA-AMINOSALICYLIC acid (PAS) is rapidly gaining recognition as an antitubercular agent, particularly when used in conjunction with streptomycin. Some authors have reported an additive therapeutic effect of the two drugs on tuberculous infections in animals (1) and on the tuberculosis organism *in vitro* (2); others have found no such effect (3, 4). While this problem remains unsettled, it seems to be well established that *Mycobacterium tuberculosis* does not develop resistance to PAS upon continued exposure to the drug or, if so, only when subjected to high concentrations for a long time and then only at a slow rate. Furthermore, development of resistance to streptomycin by *Myco. tuberculosis* is markedly reduced or is prevented when the organisms are exposed to appropriate concentrations of PAS and of streptomycin simultaneously (5). These observations appear to be substantiated by clinical experience (6, 7). Bacteriostatic action of PAS against various other bacteria has also been demonstrated, but the concentration required to effect inhibition is many times that required to inhibit *Myco. tuberculosis* (8). The physiological basis for the specificity of action of PAS against *Myco. tuberculosis* is not yet clear, but it may be related to a metabolite-antimetabolite relation between *p*-aminobenzoic acid and PAS (9, 10).

Reported studies of the concomitant action of PAS and of streptomycin on bacteria have been confined to strains of *Myco. tuberculosis*. In view of the prevalent use of streptomycin for chemotherapy of infections caused by other organisms also, it seems of interest to ascertain the effect of PAS on the action of streptomycin against some of these species. The present paper reports observations from one phase of this study in which *Escherichia coli* was used as the test organism. Since the sodium salt (NaPAS) seems to possess certain pharmaceutical and medical advantages over the acid, the salt was used in these experiments.

EXPERIMENTAL

Assay plates were prepared according to the conventional cylinder-plate technique; some plates were prepared with standard nutrient agar (controls), and others with NaPAS incorporated in the nutrient agar in concentrations ranging from 0.25×10^{-8} to 250×10^{-8} mole/ml. (0.3825 to 382.5 μg./ml.).[1] The seeded layer consisted of a 1% suspension of a strain of *E. coli* originally isolated from feces; organisms for inoculation were taken from eighteen-hour broth cultures. Streptomycin was applied in two concentrations, 100 μg./ml. and 200 μg./ml.

Following incubation at 37° for eighteen hours, each cylinder was surrounded by an inhibition zone which comprised a clear or "bactericidal" inner area surrounded by a "static" area. Presumably, in the clear zone the organisms did not grow at all, or, having grown, were lysed. In the static zone the growth of the organisms was partially suppressed, but the concentration of antibiotic was not sufficiently high or the duration of exposure was not sufficiently long to completely prevent growth. Pratt and Dufrenoy have discussed such zones and their biochemical significance at length (11).

The effectiveness of NaPAS in modifying the action of streptomycin on the test organisms was evaluated in terms of the difference between average diameters of inhibition zones (clear + static areas) produced by a given concentration of streptomycin on control plates and on plates containing NaPAS. The observations made on untreated plates were confirmed by staining with a carbolic acid-alcoholic solution of trypan blue followed by destaining with an hydro-alcoholic solution of orange G according to methods described previously (12).

RESULTS AND DISCUSSION

Two series of NaPAS and of streptomycin concentrations were studied and each was repeated several times in quadruplicate. Averaged results of the two series are presented in Table I.

The tabulated data suggest that very low concentrations of NaPAS in the medium exert a slight inhibiting effect on the action of streptomycin, while somewhat higher concentrations tend to enhance the action of streptomycin. The low concentrations of NaPAS that were employed appear to rule out the possibility that the effects observed can be

* Received October 21, 1949, from the University of California College of Pharmacy, San Francisco.
† This study was supported partially by a research grant from the Cutter Laboratories, Berkeley, Calif.

[1] Concentrations are given throughout this paper in terms of PAS.

TABLE I.—PERCENTAGE DEVIATION IN DIAMETERS OF INHIBITION ZONES ON NaPAS MEDIUM FROM DIAMETERS ON THE SAME MEDIUM WITHOUT NaPAS

Series A (Streptomycin = 100 μg./ml.)		Series B (Streptomycin = 200 μg./ml.)	
Conc. NaPAS, Moles/Ml.	Deviation from Control, %	Conc. NaPAS, Moles/Ml.	Deviation from Control, %
0.25×10^{-3}	− 1.5	0.5×10^{-3}	− 2.1
1.0×10^{-3}	− 5.6	1.0×10^{-3}	− 3.7
2.0×10^{-3}	− 2.9	2.0×10^{-3}	− 8.9
2.5×10^{-3}	+ 2.0	5.0×10^{-3}	0.0
10.0×10^{-3}	+ 3.2	10.0×10^{-3}	+ 1.9
25.0×10^{-3}	+ 5.1	50.0×10^{-3}	+10.6
50.0×10^{-3}	+ 6.4	100.0×10^{-3}	+ 9.3
100.0×10^{-3}	+ 5.6		
250.0×10^{-3}	+55.6		

attributed to a "salt effect" such as has been described for a number of ions and cations (13, 14).

In view of the well-known variations and limits of accuracy that may be expected in cylinder-plate assays, one might be tempted to attach little significance to differences as small as some of those recorded in the table. However, it should be pointed out that the experiment was repeated several times and that the same trend was observed each time. The variations were not random; quite the contrary, they followed a definite pattern. Therefore, despite the low order of magnitude of the differences, we consider them significant. Although more data would be required for adequate statistical analysis, preliminary statistical treatment confirmed these conclusions.

Although the effect of NaPAS on the cellular activities of the test organisms in the concentrations used in these experiments is unknown, there was no apparent inhibition of growth due to the drug. The minimum concentration of NaPAS that noticeably retarded growth was 1.0×10^{-3} mole/ml. (1530 μg./ml.). Therefore, it seems justifiable to conclude that the enhancement of the antibacterial action of streptomycin even in the presence of the highest concentration of NaPAS used cannot be attributed to an additive antibacterial effect of two agents acting independently.

The effects of interaction between the chemical (NaPAS and streptomycin) and the biological (organism) phases of the system studied seemed to be confined to the static areas in which the drugs permitted some growth to occur. The diameters of the clear areas in each inhibition zone were monotonously constant for a given concentration of streptomycin, regardless of the absence or presence of NaPAS in the medium. The enhancement and depression effects of NaPAS on the action of streptomycin were noted only when NaPAS was incorporated in the medium; no effect was observed when the two drugs were permitted to diffuse simultaneously from the test cylinders.

These observations showing a change from depression of streptomycin activity to enhancement, depending on the concentrations of NaPAS, afford one more example of the importance of threshold and optimal concentrations in studies of the concomitant action of two drugs on microörganisms.

The mechanism of interaction among PAS, streptomycin, and *E. coli* will be discussed in a separate report.

SUMMARY

A study was made of the effect of sodium *para*-aminosalicylic acid (NaPAS) on the activity of streptomycin *in vitro* against *Escherichia coli*. Tests were performed by the cylinder-plate technique.

NaPAS incorporated in the agar substrate in concentrations of 2×10^{-3} moles/ml. or lower inhibited the effectiveness of streptomycin against *E. coli*, whereas at higher concentrations it enhanced the effectiveness of streptomycin.

Although the magnitude of depression or enhancement was not large (except at a NaPAS concentration of 250×10^{-3} moles/ml.) the results are considered significant, since the differences noted did not vary in a random manner but, in repeated trials, always followed a definite trend. NaPAS, in concentrations used in these experiments, exerted no apparent effect on *E. coli*.

Work is in progress to elucidate the mechanisms involved in the interaction between the biological system (organism) and the drug system (NaPAS plus streptomycin).

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The Photometric Assay of Digitoxin by the Baljet Reaction*

By FRITZ NEUWALD

The color reaction of digitoxin with the Baljet reaction has been used in a quantitative study. Contrary to the statements of the literature, the results in this paper indicate the reactivity of digitoxin with the Baljet reagent follows Lambert-Beer's law.

FOR THE quantitative determination of the pure digitalis glycosides, the Baljet reaction (1), using different modifications of the Kundson-Dresbach method (2), was found to be successful in the investigations of Lendle and Schmelzer (3), Hauschild (4), Neumann (5), Hagemeyer (6), Bell and Krantz (7), Anderson and Chen (8), and Neuwald (9). The Bell and Krantz method has been made the basis for method I, Digitoxin Colorimetric Controls, in the U. S. P. XIII. In a series of papers Bell and Krantz (7, 10–12) have shown that the reaction of digitoxin and other pure digitalis glycosides with alkaline trinitrophenol reagent does not follow Lambert-Beer's law. Therefore U. S. P. XIII directs the preparation of a standard digitoxin graph instead of the calculation of a standard digitoxin factor which is possible if Beer's law is fulfilled. In addition, Neumann (5) has stated that the reaction of digitalis glycosides with the Baljet reagent does not exactly follow Beer's law. In the studies of Hagemeyer (6) it is obvious that the degrees of extinction do not lineally follow the increase of the concentration of digitoxin solutions. Canbäck (13) pointed out that the invalidity of Beer's law is a disadvantage of the modified Knudson-Dresbach method. In my investigations of photometric assay of digitalis drugs (9), I have also determined the quantitative behavior of numerous pure digitalis glycosides and aglucones in the Knudson-Dresbach method, modified by using a photometer with a spectral filter of 490 mμ (maximum transmission). Contrary to the above statements of different investigators, I have found that the reaction of digitoxin and other cardiotonic glycosides and aglucones with the Baljet reagent fulfills Lambert-Beer's law. By this means the extinction E of a test solution is proportionate to the light path and, if light paths are equal, directly proportionate to the concentration. Therefore the readings of extinction which are obtained in different light paths can be calculated in a light path of 10 mm. and we receive the extinction coefficient k which, multiplied with a factor (standard factor), shows the concentration of the colored substance. This paper will describe and discuss the reason for the validity of the different results of the Lambert-Beer law.

EXPERIMENTAL

Due to the kindness of Dozent Dr. R. Tschesche (Biochemisches Institut der Universität Hamburg), I was fortunate in obtaining different samples of digitoxin especially prepared for research studies. For purposes of comparison I have used a Digitoxin (Merck). The experimental method employed was a modified Knudson-Dresbach method. The instrument used was a Zeiss-Pulfrich Photometer with different spectral filters and absorption cells with different light paths. Stock solutions of the various samples of digitoxin in methanol *pro analysi* (Merck) were prepared and, by proper dilution with the same solvent, solutions containing 5, 10, 15, and 20 mg., respectively, per 100 cc. were obtained for each sample. To 10 cc. of each solution, 10 cc. of freshly prepared alkaline picrate reagent was added, the time noted, and the solution mixed well.

The reagent was made as follows: 95 cc. of 1% purified trinitrophenol solution was mixed with 5 cc. of 10% sodium hydroxide solution according to the original presciption of Knudson and Dresbach (2). After thirty minutes the extinction coefficient k was determined in the photometer at a temperature of 20° ± 1°. The blank used for the determination was a mixture of equal volumes of methanol, analytical grade, (Merck) and the trinitrophenol reagent. The accuracy of the method is about ±2%.

$$\text{Extinction coefficient } k = \frac{\text{extinction}}{\text{cm. of light path}}$$

Selection of the Suitable Spectral Filter.—With regard to the selection of the most suitable spectral filter, the photometric principle is observed and the measurements are carried out in the region of an absorption maximum. As described previously for digitoxigenin (9), the absorption curve of digitoxin in Fig. 1 is prepared by plotting the wave length in millimicrons of the effective filter gravity of the Zeiss spectral filters against the logarithm of extinction of a digitoxin solution (10 mg. %) in a 10-mm. absorption cell. With regard to the picrate reagent, the effective filter gravities and the transmission regions of the different S filters were measured with a spectrometer.[1] The reading of extinc-

* Received June 3, 1949, from the Staatsinstitut für Angewandte Botanik, Universität Hamburg, Germany.

[1] I am indebted to Dozent Dr. E. David, Physikalisches Institut der Universität Hamburg, for helpful criticism.

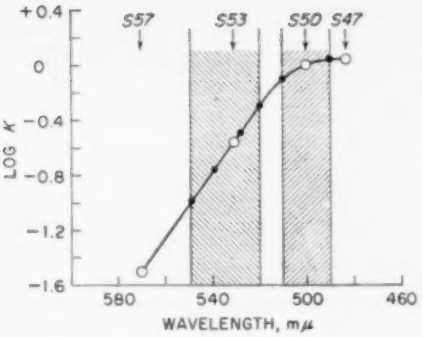

Figure 1.

Log k	Wavelength	
−1.5	570 mμ	(S 57)
−0.96	550 mμ	
−0.74	540 mμ	
−0.52	533 mμ	(S 53)
−0.49	530 mμ	
−0.29	520 mμ	
−0.10	510 mμ	
0	500 mμ	(S 50)
+0.04	490 mμ	
+0.04	483 mμ	(S 47)

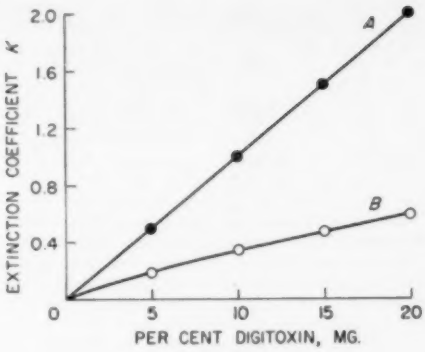

Figure 2.

	Digitoxin, Mg. %	Extinction Coefficient k
Curve *A*:	5	0.50
	10	1.00
	15	1.50
	20	1.99
Curve *B*:	5	0.19
	10	0.33
	15	0.47
	20	0.59

tion is impossible at S 43. The absorption curve shows that filter S 53 (maximum transmission at 529 mμ; effective filter gravity, 533 mμ) is not suitable at all, because the transmission region (520–550 mμ) is situated in the steepest part of the curve. Because filter S 47 (effective filter gravity, 483 mμ) is also almost unsuitable, due to the very strong absorption of the Baljet reagent, the filter S 50 (effective filter gravity, 500 mμ; maximum transmission at 490 mμ) is the most suitable. The transmission region of this spectral filter (490–510 mμ) is situated nearly in the peak absorption. This result agrees with that of digitoxigenin previously described (9).

Effect of Various Spectral Filters.—Extinction coefficient-concentration curves prepared from the samples of digitoxin all showed good agreement with that obtained from the digitoxin sample (Merck) which was accepted as standard. The curves in Fig. 2 are prepared by plotting the four points of concentration against the corresponding mean k values of all samples. The original data of k values obtained from the various digitoxin samples are given in Tables I and II. Curve *A* was obtained with filter S 50 and the 5- and 10-mm. absorption cells. Curve *B* is the corresponding curve when filter S 53 and only the 10-mm. cell are used. The curve *A* is a straight line and it is seen that the absorption produced by the digitoxin solutions follows Lambert-Beer's law closely.

The digitoxin factor

$$F = \frac{\text{concentration (mg. \%)}}{\text{extinction coefficient } k}$$

is 10 under these conditions. On the contrary, curve *B* is smooth, and it is obvious that there is a marked divergence from Beer's law in the behavior of the digitoxin solutions by using a green filter with peak transmission at 529 mμ. This divergence, however, is only apparent, since using different absorption cells it has been found that a solution of 5 mg. % in a 30-mm. cell has the same reading as a 15 mg. % solution in a 10-mm. cell and a 10 mg. % solution in a 20-mm. cell, the same as a 20 mg. % solution in a 10-mm. absorption cell. Bell and Krantz (11) have observed this also, but have concluded: "it appears that the colored substance produced in the Baljet test follows Beer's law although the reaction which produces the color does not."

TABLE I.—EXTINCTION COEFFICIENT k VALUES FOR SOLUTIONS PREPARED FROM SEVERAL DIGITOXIN SAMPLES USING DIFFERENT CONCENTRATIONS AND FILTER S 50

Sample	5 Mg. %	10 Mg. %	15 Mg. %	20 Mg. %
1	0.49	1.01	1.51	2.01
2	0.49	0.98	1.47	1.96
3	0.50	1.00	1.50	1.99
4	0.50	1.00	1.50	1.98
S41699 (Merck)	0.50	1.00	1.52	2.00

TABLE II.—EXTRACTION COEFFICIENT k VALUES FOR SOLUTIONS PREPARED FROM SEVERAL DIGITOXIN SAMPLES USING DIFFERENT CONCENTRATIONS AND FILTER S 53

Sample	5 Mg. %	10 Mg. %	15 Mg. %	20 Mg. %
1	0.19	0.34	0.47	0.59
2	0.19	0.34	0.46	0.58
3	0.19	0.33	0.47	0.59
4	0.19	0.33	0.47	0.58
S41699 (Merck)	0.19	0.33	0.47	0.59

TABLE III

Expt.	Composition of Baljet Reagent	Extinction
1	2 Gm. trinitrophenol/50 cc. methanol + 0.5 Gm. NaOH/50 cc. H_2O	0.66
2	1 Gm. trinitrophenol/50 cc. methanol + 0.5 Gm. NaOH/50 cc. H_2O	0.78
3	1 Gm. trinitrophenol/90 cc. H_2O + 1 Gm. NaOH/10 cc. H_2O	0.92
4	0.5 Gm. trinitrophenol/90 cc. H_2O + 1 Gm. NaOH/10 cc. H_2O	0.96
5	0.95 Gm. trinitrophenol/95 cc. H_2O + 0.5 Gm. NaOH/5 cc. H_2O	1.00

Effect of Various Compositions of the Baljet Reaction.—Table III shows typical data for five different experiments. To study the influence of various concentrations of trinitrophenol and sodium hydroxide, and of water and methanol as solvents for trinitrophenol, experiments have been carried out with the same stock solution of digitoxin containing 10 mg. of the glycoside per 100 cc. methanol in each determination, using a 10-mm. cell, and readings were made after thirty minutes. It will be recognized that in Experiment 1 the Baljet reagent is prepared according to the directions of the U. S. P. XIII, while in Experiment 2 the trinitrophenol concentration has been reduced 50%. In Experiments 3, 4, and 5, the solvent for trinitrophenol was water instead of methanol. The composition of the reagent in Experiment 5 agrees with the directions of Knudson and Dresbach and it is obvious that under these conditions the color intensity reaches a maximum value.

DISCUSSION

The validity of Lambert-Beer's law may only be expected when external conditions are constant. To these external conditions the monochromasy of the used light belongs—i.e., by not using sufficient monochromatic light an apparent invalidity of Beer's law may appear, the influence of which is not frequently recognized. The seeming invalidity of Beer's law caused by nonmonochromasy can be eliminated if the measurements are carried out at constant extinction, i.e., at constant product: concentration × light path.

This way of determining the concentration of an unknown solution is not possible, however, in the photometers generally used. An insufficient monochromasy does not affect the validity of Beer's law if the absorption coefficient is constant within the transmission region. This coefficient is nearly constant at a maximum absorption and therefore the maximum transmission of the used filter should be identical with the maximum absorption of the colored substance (14). The filter S 50, together with the Baljet reagent cutting out the region 490–510 mμ with the effective filter gravity 500 mμ, suitably fulfills this condition, while with the regions 520–550 mμ the filter S 53 does not. The observations of this paper show that Beer's law is valid and the reason for the different results on the validity of Lambert-Beer's law in literature is the use of insufficient monochromatic light.

SUMMARY

The results of a quantitative study of the color reaction of digitoxin toward the Baljet reagent have been presented and discussed. It has been found that the reaction follows Lambert-Beer's law if a spectral filter giving a maximum absorption for the colored substances is used. A suitable filter for digitoxin colorimetric control has a maximum transmission at about 490 mμ. The use of an alkaline trinitrophenol reagent according to the directions of Knudson-Dresbach has been proved favorable.

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Colorimetric Determination of Histamine in Histamine Phosphate Injection, U. S. P.*

By W. J. MADER, H. S. STERNE, Jr., J. ROSIN, and H. A. FREDIANI

Folin's colorimetric method for the determination of amino acids has been found applicable to the analysis of histamine phosphate injection, U. S. P. A color is developed by treating histamine with 1,2-naphthoquinone-4-sodium sulfonate which obeys Beer's law within the range indicated. Optimum conditions for this color reaction and measurement have been defined.

THE PRECISE control of histamine dosage is important because of its marked vasoconstrictor activity (1) and its use as a desensitizer in certain allergic conditions. Biological assays such as those utilizing contraction of isolated strips of intestine (2) are not adapted to quantitative determinations. The colorimetric method as originally recommended by Gibauer-Fulnegg (3) for the estimation of histamine and as modified by Rosenthaler and Tabor (4) for determining histamine in urine and tissue extracts has been shown to be precise, but for routine analysis of relatively simpler pharmacological preparations this method is tedious and time-consuming. Moreover, phenol which is usually present as a preservative in the parenteral solutions, interferes. For adequate control of such preparations, it was deemed advisable to develop a more rapid procedure that embodies the requisite sensitivity and precision.

EXPERIMENTAL

A general colorimetric method for amino acids has been proposed by Folin (5) and modified by France, Russell and Wilhelmi (6). In this method, 1,2-naphthoquinone-4-sodium sulfonate is permitted to react with the amino acid with the resultant formation of a striking deep red color. For histamine, the color follows Beer's law in the concentration range of 0 to 0.08 mg. The method is rapid and reproducible (standard deviation $\pm 3\%$). Glycerin and phenol, which are present in commercial histamine phosphate solutions, do not interfere with the determination. Ammonia and certain nitrogenous bases, such as the alkaloids and aminobenzenes give positive reactions with the reagent, but their presence is not encountered in histamine injections.

Reagents:

0.5% 1,2-naphthoquinone-4-sodium sulfonate: Prepared from pure material daily. 500 mg. is dissolved in water and diluted to 100 ml.

Acid Formaldehyde: To 45 ml. of 1 *N* hydrochloric acid add 10 ml. of glacial acetic acid, 0.5 ml. of 40% formaldehyde and dilute to 80 ml. with distilled water.

Sodium Tetraborate (borax): Dissolve 1 Gm. in 100 ml. of water.

Sodium Thiosulfate: 0.1 *N* prepared by dissolving 24.819 Gm. $Na_2S_2O_3 \cdot 5H_2O$ in a liter of water.

Standard Histamine: Dissolve 33 mg. of dry histamine dihydrochloride in water and dilute to 100 ml. Dilute 10 ml. of this solution to 200 ml. Each milliliter of this solution is equivalent to 0.01 mg. of histamine.

PROCEDURE

Dilute the unknown solution so that 5 ml. contain 0.035–0.055 mg. of histamine base or an equivalent weight of histamine acid phosphate.

To one of three 10-ml. volumetric flasks, add 5 ml. of the unknown, to the second 5 ml. of the standard histamine and to the third 5 ml. of water. Add 1 ml. of sodium tetraborate solution to each, followed by 1 ml. of the 1,2-naphthoquinone-4-sodium sulfonate solution. Place the flasks in a boiling water bath for ten minutes then immerse in a cold water bath (4–10°) for five minutes. Add 1 ml. of the acid formaldehyde and then 1 ml. of 0.1 *N* sodium thiosulfate to decolorize the excess reagent. Make up to volume, mix well, and set aside for thirty minutes. Transfer to matched 10-ml. absorption tubes. Read the color in a suitable colorimeter at a wave length of 425–520 mμ, preferably 460. Figure 1 shows an absorption curve obtained on a Cary recording spectrophotometer showing a maximum of 463 mμ. In this work we used a Klett colorimeter with a No. 52 filter. The concentration of histamine is calculated as follows:

(*a*) $\dfrac{\text{Sample density}}{\text{Standard density}} \times \text{concentration of standard} = \text{Histamine}$

(*b*) Histamine $\times$ 2.76 = Histamine Acid Phosphate

DISCUSSION

As may be seen from Fig. 2, the color developed shows close adherence to Beer's law. For this reason it is possible to use such a calibration curve instead of preparing simultaneous standard histamine solutions each time the test is carried out. In the analysis of known solutions prepared by the use of reagent grade histamine hydrochloride, which had been previously assayed by formal titration and phase purity techniques, recoveries of $100\% \pm 3\%$ were obtained. The analysis of histamine phosphate injections obtained on the open market are reported in Table I. Because one of the available samples indicated 130% of the labeled histamine content, it was deemed advisable to confirm the analyses by in-

* Received April 30, 1949, from the Chemical Control Division, Merck and Co., Inc. Rahway, N. J.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

TABLE I.—ANALYSIS OF HISTAMINE PHOSPHATE INJECTIONS OBTAINED ON THE OPEN MARKET

Manufacturer	Histamine Phosphate			
	Labeled Content, mg./ml.	Found Colorimetrically, mg./ml.	Found from Nitrogen, mg./ml.	Found from Phosphate, mg./ml.
A	1	1.07	0.96	1.02
B	0.55	0.72	...	0.8
B	0.55	0.61	0.60	...
Synthetic mixture containing histamine, phosphoric acid and phenol	1.00	1.00 ± 0.03	1.01	...

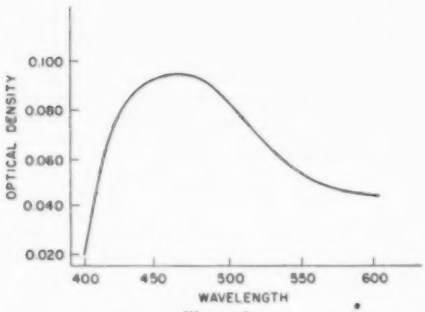

Figure 1

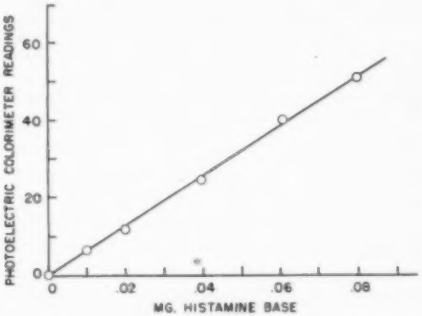

Figure 2

dependent means. For this reason the samples were tested for nitrogen content by a micro-Kjeldahl determination, using the apparatus of Kirk (7) and by the photometric phosphate method of Eddy and De Eds (8). The reasonably good agreement by these independent methods collaborated the original findings.

STUDY OF VARIABLES

Such variables in the technique as time, temperature, amount of reagents, and the most optimum wave length for the measurement of color have been studied.

The reagent used in this work was prepared by the method recommended by Folin (5) and was recrystallized from borax solution. This reagent was found to be stable in aqueous solution for periods up to eight hours. Commercial 1,2-naphthoquinone-4-sodium sulfonate was found unstable unless recrystallized. Several substances, such as sodium borate, ascorbic acid, sodium sulfite and hydroquinone, have been added to the solution of the reagent in unsuccessful attempts to increase the stability.

SUMMARY

A colorimetric photoelectric method has been developed whereby the U. S. P. XIII histamine phosphate injection may be assayed for histamine by comparison with standard histamine. The new method is more precise than existing methods for determining histamine in simpler pharmacological preparations.

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Estrogenic Substances from Plants: I. Glycyrrhiza*,†

By CHRISTOPHER H. COSTELLO‡ and E. V. LYNN§

Licorice root was found to contain an estrogenic hormone in appreciable quantity, and an efficient process was devised for its concentration. Spectrophotometric experiments indicated that it is a steroid, and other evidence points to its identity with estriol. An antihormonal principle may also be present. Asclepias, helonias, and aletris were also found to be estrogenic.

THE greatly increased employment of estrogens in therapy has recently caused scientists to search for more available sources than previously used animal excretions. The so-called synthetic compounds, such as diethylstilbestrol, meet the need for certain purposes, but are far inferior to the natural hormones in potency and broadness of application. These natural substances, chiefly estrone, estriol, and estradiol, are usually obtained from the urine of pregnant animals, and naturally the cost of extraction makes them very expensive. It is known, however, that the same or physiologically related compounds are present in a great variety of vegetable materials, and it is possible these could be isolated at a great reduction in price. Fellner (2) found them in wheat, oats, and rice in 1925. A year later Dohrn, *et al.*, demonstrated (3) that they are present in yeast cells, sugar beet seeds, potatoes, parsley roots, cherries, plums, etc., and the name "tokokin" was suggested to designate this variety of estrogen, later also called "thelykinin." In 1927, Loewe, *et al.*, found them (4) in the flowering parts of various plants, especially yellow pond roses and pussy willows. Walker and Janney, in 1930, showed (6) that they are present in many leaves, such as those of alder and rhubarb, and in the catkins of alder. They have also been found in the flowers of elderberry, linden, and nettles by Much (8), in garlic by Glaser and Drobnik (12), and by Hassan and Wafa (16) in date palm pollen.

Only in three cases have the estrogenic substances been isolated and identified. In the others the presence has been shown by the vaginal smear method of Allen and Doisy (1). In 1933, Butenandt and Jacobi (9) extracted estrone from palm kernel residue in a yield of 18 mg. from 50 Kg. In the same year, Skarzynski (10) succeeded in isolating estriol from pussy willows, 5.5 mg. from 65 Kg. of raw material. In 1947, Hassan and Wafa (16) isolated a substance from the date palm pollen and identified it as estrone. It might be noted also that estrogenic compounds have been made synthetically from plant sources; for example, Brandt and Ross (17) have prepared such a substance from podocarpic acid, which occurs in the wood of *Dacrydium cupressinum*.

EXPERIMENTAL

The present work was begun in 1946 during a pharmacological and chemical study of a proprietary mixture used in the treatment of the symptoms associated with dysmenorrhea and the menopause. Pharmacological and clinical bases existed for its value in the former, but its use in the menopause was essentially empirical, although some clinical evidence has since been acquired. The mixture consisted of fluidextracts of several drugs, some of which are official. Early experiments indicated that the total solids give a typical estrogenic response, but it soon became apparent that the material must be obtained in a more concentrated form to obtain quantitative results in the standard bioassay, which is essentially that of the Allen and Doisy (1), vaginal smear technique.

The method for fractional separation was a modification of that used by Pincus (15), for the isolation of phenols from urine. The dry, dealcoholized, extract was boiled with 15 parts of concentrated hydrochloric acid for seven minutes and filtered from the resins. The aqueous solution was extracted with ether, which was then washed with a saturated solution of sodium carbonate and with water. The ether solution was then extracted with 2 *N* sodium hydroxide, and the alkaline solution, after acidifying, was re-extracted with ether, and the solvent was evaporated to give a "total phenol" fraction.

The final product was assayed by us and by two commercial laboratories. The results were somewhat variable in amount, apparently depending upon the particular lot of material, but all showed considerable activity, and the highest results were submitted by the commercial laboratories. In terms of those from pure estrone as unity, they may be listed as 0.0005, 0.1, 0.0043, and 0.0053.

* Received September 29, 1949, from the Massachusetts College of Pharmacy, Boston.

† The authors wish to acknowledge the technical assistance of Mr. Calvin L. Butler and Miss Lucille E. Mullen of the Lydia E. Pinkham Research Laboratories in the bioassays and of Professor Helen S. French of the Chemistry Dept., Wellesley College, in the spectrophotometric analyses. The authors are indebted to the Lydia E. Pinkham Medicine Co. for supplying raw materials and to Parke Davis and Co. for supplying the crystalline estriol used in this investigation.

‡ Abstracted from a thesis presented to the Graduate Faculty of the College by Christopher H. Costello in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address of Dr. Costello: The Columbus Pharmacal Company, Columbus, Ohio.

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The total phenol portion was further separated into individual fractions of the natural estrogens. It was dissolved in benzene, and this solution was extracted with 0.3 *M* sodium carbonate. The residual benzene, which by the Pincus method contains estrone and estradiol, had little or no activity, but the acidified aqueous solution yielded to ether a very potent fraction, presumably containing estriol if present. Doses of 0.5 to 0.8 mg. gave estrus in 50% of the rats, a potency equal to 0.002 on the basis of 1 for estrone.

In order to determine which of the ingredients in the original mixture is responsible for the activity, an alcoholic extract of each was processed to the total phenol stage and submitted to bioassay. The drugs so treated included Glycyrrhiza (Licorice) U. S. P., Asclepias N. F. V, Helonias N. F. V, Aletris N. F. VII, Cimicifuga N. F. VIII, Senecio N. F. V, Gentian U. S. P., Matricaria N. F. VIII, and Taraxacum N. F. VIII. Of these the first four exhibited great activity while others, including especially senecio and taraxacum, produced an insignificant degree of estrus. Glycyrrhiza appeared to be the most consistently and strongly active and was selected for immediate further study. The work reported in this investigation is, therefore, confined mostly to establishing the identity of the estrogenic material in licorice, to determining the amount present, and to devising a method for its extraction.

The yield of total phenol from 2 Kg. of finely powdered licorice root was 12.2 Gm., or 0.61%. As might be expected, the potency of this fraction varied from lot to lot; the most active sample had a maximum of 0.018 that of estrone. All of them evidently contained an antihormonal principle, because larger doses gave smaller activity, as illustrated by the following typical example:

Dose Micrograms	Per Cent Positive
30	80.0
60	82.1
120	34.4
200	0.0

If it is assumed that the low dosage at which significant activity was measured represents the effective dosage, the results show that 100 parts of the fraction is equal to 1.87 parts of estrone, a ratio of 1:0.0187. The figures are of necessity approximate in view of the peculiar effect of the increased dosage. It may be assumed that the fraction probably contains material that inhibits the activity on the test animals, and that this inhibiting result becomes almost absolute at the higher dosage. These phenomena were confirmed by tests in other laboratories. In any event, it was concluded that a more selective method of extraction was required to determine the true activity.

Extraction.—Separate portions of one lot of licorice were extracted with the following solvents: 95% ethyl alcohol; 99% isopropanol; benzene; ether; acetone; aqueous and alcoholic solutions of sodium hydroxide; and water. Soxhlet extraction was employed for the undiluted solvents and percolation for the diluted solvents, except for the alkaline extracts which were made by digestion. All but the last were assayed without further processing except to remove the solvent and take up the residues with 95% alcohol before injection. Of all the solvents, the 95% alcoholic extracts were the most potent and were the easiest to handle; they produced estrus in the test animals in doses representing 0.34 to 1.2 Gm. of the whole drug.

Although continuous extraction was preferable because of a higher yield of total product, a sufficiently large apparatus was not available. Instead, 100 pounds of licorice root was extracted by circulatory maceration and percolation, using alcohol as the solvent. The latter was removed by vacuum distillation, and the extract was dried in a vacuum oven at low temperature to give 10 pounds of dry product. In the meantime, several 5-Kg. batches of other lots were extracted in a Soxhlet apparatus with the same solvent, giving about 12% of dry product. The raw material in each case was identified as authentic by Professor Heber W. Youngken of the Massachusetts College of Pharmacy.

For the further isolation of the potent ingredient, many methods were tried and several of these were modified in different ways. Space does not permit a complete description for all of these, but the chief procedures can be outlined.

Modified Pincus Method.—The basic procedure has already been described, but many variations were made in order to obtain more potent fractions. In one instance an attempt was made to purify the total phenols by precipitating them from concentrated alcoholic solution by the addition of excess water. This was repeated until there was no further rise in the melting point of the dried precipitate. This yielded a yellow, amorphous powder that melted at 194–196° and represented 36% of the total phenols. It was found to have the same potency in 20- to 40-mg. doses as the total phenols from which it was derived. Another laboratory, using much smaller doses, reported that the product had 0.001 the activity of estrone or one twentieth that of the total phenol fraction. Here again, it was found that above a certain dosage activity declined to zero; for example, responses were obtained at 0.5 mg. but not at 1 mg.

In attempts to recrystallize the material, boiling water was found to give yellow, microscopic needles which, after drying, melted at 189.4–190.4°. They were not identical with the original and are believed to be a hydrolytic product.

In another experiment it was found that much of the active agent is removed by washing with sodium carbonate. The washings were acidified to a *p*H of 2.5 and extracted with ether, and the recovered extract was assayed. It was found that a large portion of the total estrogenic activity of the drug resided in this fraction, sometimes exceeding the yield from the entire process.

Commercial licorice mass, using the modified Pincus method, yielded a total phenol fraction in smaller yield than from our extract, but estrogenic in 30-mg. doses. On the other hand, commercial Glycyrrhiza Extract U. S. P. gave inactive, aqueous, and alcoholic products. Because of the great variation in potency already found, this does not mean that all extracts are inactive.

Modified Skarzynski Method.—Since preliminary experiments had shown that estriol is the probable agent, we also tried the method used by Skarzynski (10) in extracting this substance from pussy willows. The chief differences were in omitting the

hydrolysis and in liberating the phenols from alkali by treatment with carbon dioxide. Again, several modifications were made, as well as combinations with the method of Pincus.

From these experiments, it was found that the hydrolysis is unnecessary for the licorice extract, that the potent ingredient is widely distributed in the various fractions, and that the previously discarded resin contained even more than the non-resin portion. This resin was of reddish brown color and pilular consistency and quite surprisingly dissolved completely in 2 *N* sodium hydroxide. It represented about 0.1% of the original drug and was active in 10-mg. doses.

Using this method of separation, we again obtained the substance melting at 194–196°. In addition, a compound was separated in colorless crystals from the final ether extract. This melted at 119–122° and proved to be benzoic acid.

Revised Method.—Using the experience of previous separations, a method was devised to increase the yield of active ingredient and concentrate it in one fraction. In this the alcoholic residue was extracted with peroxide-free, absolute ether that had been redistilled. This removed 46% of the total solids, the insoluble residue being inactive. The ethereal solution was extracted with 2 *N* sodium hydroxide, and the alkaline solution was acidified to *p*H 2.5 to precipitate resins that were discarded. The aqueous solution was exhausted with ether, and the solution so obtained was washed with water, dried, and evaporated in a vacuum to produce the total phenols. The dark brown product was found, in doses of 20 mg., to produce a greater degree of estrus than 0.001 mg. of estrone. It was further purified by distilling off with steam about 4% of inactive material.

Another portion of the phenols was treated with benzene for the isolation of estriol. This removed about 60% of the material as a dark brown semisolid, accompanied by a small amount of crystals that proved to be benzoic acid. The sodium carbonate extract of the benzene phase was acidified and yielded to ether a dark brown semisolid that was active in 20-mg. doses. The result seemed to show that benzene does not remove all of the estrogen, as previously observed by Bachman and Pettit (13) for estriol.

Modified Marrian Method.—The original Marrian (7) process was devised to isolate estriol from urine. After some modification, it proved to be the most effective in that it gave a larger amount of total phenols that had equal activity. The ethereal extract of the original material was heated on a water bath for thirty minutes with 5% potassium hydroxide, after which the solution was saturated with carbon dioxide and extracted with ether. The dried, ethereal extract was treated with boiling acetone and, after cooling to −10° and maintaining this temperature for twelve hours, filtered, and the acetone was removed by vacuum distillation. The active residue was taken up in 50% alcohol, the solvent was removed by suction, and the residue was dissolved in ether containing some alcohol. The ethereal solution was extracted with 5% potassium hydroxide, and the alkaline solution was acidified with hydrochloric acid and exhausted with ether. The ethereal solution, after being dried and evaporated in a vacuum, yielded a dark brown solid that represented 0.075% of the original drug and gave estrus in 75% of the test animals in 20-mg. doses.

For the further treatment of this fraction, we used a modification devised by Friedgood and Garst (18). It was dissolved in a minimum of alcohol and diluted with 200 cc. of benzene; in previous operations it had been learned that benzene alone does not dissolve all of the estrogens in the phenol fraction. The benzene solution was washed with 9% sodium bicarbonate, which removed large amounts of pigment and acidic materials, as well as a small quantity of the estrogen. It was then extracted with 0.075 *M* secondary sodium phosphate, the separated aqueous solution was acidified with 6 *N* sulfuric acid, and the estrogenic materials were removed by ether. We were able to separate by this method a dark brown extract representing 0.035% of the original licorice and having an activity such that 2 to 3 mg. produced estrus in the test animals. This is the most potent fraction thus far separated from licorice by any method.

Since it is known that estrone and estradiol lose much of their activity when taken orally, whereas estriol retains most of it, it was decided to feed a group of ovariectomized rats solutions of this fraction by stomach tube, and to observe its estrogenic activity, if any. Doses of 5 mg. administered in this manner produced a complete estrogenic response in these test animals, showing that no appreciable inactivation had taken place.

Absorption Spectra.—The active material was compared with estriol chiefly by means of absorption spectra. Calculations based on the assays have shown that some 200 pounds of licorice would need to be processed in order to isolate a few milligrams of any sterol. Several investigators (14, 18, 19) have shown that the ultraviolet absorptions of estrone, estradiol, and estriol are very similar, in that minimum densities occur between 248 and 252 mμ and maximum densities occur between 280 and 282 mμ. Friedgood and Garst (18), have demonstrated that, despite this similarity, all three may be detected and determined accurately by application of the sharply separative chemical procedures we have used.

The apparatus used in this study was a Hilger medium quartz spectograph with a Spekker photometer, and tungsten steel electrodes furnished the spark. The solvent employed was 95% ethanol in all cases. One cubic centimeter tubes were used throughout the tests. The absorption spectra were recorded on Eastman "40" 4 x 10-inch photographic plates. The region from 220 to 380 mμ was investigated in these experiments. The ordinates were expressed in terms of density, i.e., $E = \log_{10}(I_0/I_x)$ = density, where I_x is the intensity of the light transmitted by the sample and I_0 the intensity of the incident beam. From these data, absorption curves were constructed.

The absorption curve of estriol was first determined. A solution containing 4 γ/ml. produced a curve in essential agreement with the literature, with minimum density at 252 mμ and maximum density at 280 mμ (curve *A*, Fig. 1).

The first licorice fraction submitted to this method of analysis was a total phenol fraction obtained by the revised method from the ether extract of the original material. This fraction was found pre-

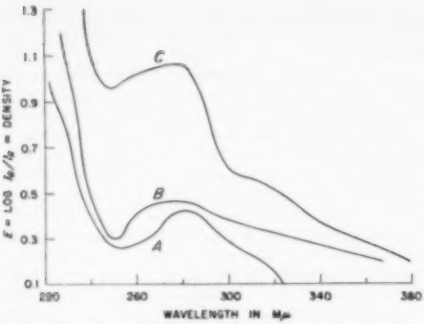

Fig. 1.—Comparison of absorption spectra curves of estriol with those of active fractions of licorice. *A*, estriol; *B*, total phenol fraction licorice; C, estriol fraction licorice.

viously to be estrogenic in doses of 10 mg. Because of the highly colored nature of its solution in alcohol, it was necessary to dilute 1:50,000 before sufficient light passed through to observe the absorption. The curve thus obtained agreed closely with that of the estriol sample previously tested, not only in maximum and minimum densities, but in general outline as well (curve *B*, Fig. 1). The minimum density occurred at 250 mμ and the maximum density was found at 280 mμ.

Since the total phenol fraction could contain estrone and estradiol as well as estriol and other sterols of similar composition, it was separated by methods previously described into two fractions. The total estrogenic activity was found on assay to reside in the estriol fraction. It was necessary to dilute 1:12,000 with alcohol in order to obtain the absorption curve (curve *C*, Fig. 1). The minimum density was found to be at 249 mμ and the maximum density occurred at approximately 280 mμ, both of these being in agreement with curves from estriol and from the total phenol.

Antihormonal Principle.—Several investigators of hormones have presented evidence for the existence of natural counteracting substances, and some have postulated that every hormone has its corresponding antihormone. Previously in this paper we have recorded results of biological assays of licorice fractions that seemed to corroborate such opinions. In these the activity seemed to decrease with larger doses up to a certain point and then increased as the dosage was raised.

The results with absorption spectra gave us further possible evidence of this, if we should assume that estriol is the active substance. In their study of the effect of concentration of estrone, estradiol, and estriol, Friedgood and Garst (18) found that the relation between density and concentration followed Beer's law, and that the concentration of estrogen could be determined within limits by determining the density at the peak of 280 mμ. When the three curves are superimposed as in Fig. 1, that for the diluted phenol fraction (curve *B*) would indicate nearly 100 times the activity that could be calculated by the dilution. This is on the basis that 10 mg. of the undiluted sample had the same estrogenic response as 8 μg. of estriol. In other words, the activity is much greater than would be indicated by biological assay.

Background materials could be responsible for some of this increased density, but past experience indicates that such a radical increase could not be expected from impurities, especially since the maximum and minimum densities and the general shape of the curve remain essentially the same as with the pure sterol.

Non-Estrogenic Crystalline Material.—In the foregoing several references have been made to separation of a crystalline, nonestrogenic substance from licorice. This was a pale yellow to colorless, crystalline compound melting at 194–196.1°, and was found usually in the total phenol fraction. On boiling this material with water and cooling, yellow, microscopical crystals appeared in feathery aggregates, melting at 189.4–190.4°. Chemical investigation of these two compounds led us to believe that they are identical with the compounds $C_{26}H_{21}O_9$ isolated from Chinese licorice root by Houseman and Swift (5) in 1929, and the hydrolysis product $C_{18}H_{24}O_4$. The latter was thought by them to be closely related to hydroxy-α-lapachone.

SUMMARY

1. The ingredients of a proprietary formula that is estrogenic was found to owe this property to glycyrrhiza, asclepias, helonias, and aletris, but chiefly the first.

2. Glycyrrhiza was found to contain in significant amounts an estrogenic substance that could most practically be isolated by a modification of the Marrian method, originally devised to separate estriol from urine. Ultraviolet spectrophotometric analysis of the extract gave absorption spectra similar to those from the steroid estrogens, but the presence of estrone and estradiol were excluded by the method of extraction. Some evidence was also obtained for the presence of an antihormone.

3. The samples of glycyrrhiza also contained benzoic acid and two nonestrogenic compounds, which are probably identical with those isolated by Houseman and Swift in 1929.

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Studies on *Mahonia* Genus. Part I. The Alkaloids of *M. Griffithii* Takeda*

By R. CHATTERJEE and M. P. GUHA†

Five alkaloids, oxyacanthine, berbamine, berberine, neprotine, and palmatine, have been isolated and identified from *Mahonia Griffithii* Takeda obtained from Bhutan.

BOTANICALLY *Mahonia* was treated for a long time as a section of *Berberis* (Family *Berberidaceae*). Fedde (1) in later years examined the genus *Mahonia* from systematic, anatomical, and morphological points of view and was of the opinion that *Mahonia* should be treated as quite distinct a genus from *Berberis*. Many alkaloids which are known to occur in several species of *Berberis* have also been isolated from the species of *Mahonia* in India and other countries (2). The chemical investigation of the *Berberis* plants dates back to the year 1837, when Buchner (3) isolated berberine from the common European species, *B. vulgaris*. With the recognition of berberine in medicine, other sources of berberine (2) were looked for, and the first species of *Mahonia* which was examined was an American plant, *M. aquifolium*, from which Parsons[‡] (4) isolated, in 1882, the alkaloids oxyacanthine and berberine. In 1891, Rüdel re-examined the plant and identified the alkaloid berbamine which was associated with oxyacanthine (5). Since then several species of *Berberis* have been examined chemically, but there is no record of *Mahonia* species being investigated until 1932, when Santos and his collaborators (6) obtained berberine, jatrorrhizine, and shobakunine from *M. philippensis*. Six years later two species of *Mahonia* from Texas, *M. trifoliolata* and *M. Swaseyi*, were examined to obtain a fairly large percentage of berberine (7). In 1944, Chatterjee isolated two new alkaloids, umbellatine and neprotine, from a Himalayan species, *M. napalensis* (8).

In the present paper, the isolation and identification of five alkaloids from the bark of *M. Griffithii* have been described. The plant material was obtained from Bhutan, its place of occurrence, by courtesy of Mr. B. Pradhan, Forest Manager, Sikkim State.

* Received on August 30, 1949, from ICI Research Fellow's Laboratory, Department of Chemistry, University College of Science and Technology, 92, Upper Circular Road, Calcutta-9, India.

† Our thanks are due to Dr. N. K. Sen, Head of the Department of Chemistry, Presidency College, Calcutta, for affording us laboratory facilities.

EXPERIMENTAL

Finely powdered bark (1000 Gm.) was extracted completely with warm ethanol (95%). The ethanol was removed under reduced pressure and the sticky residue was treated with iced water (500 cc.) and filtered three times to get rid of the resinous matter. The clear dark brown solution was cooled and made alkaline with ammonia (25%), when a sticky amorphous solid separated, and the mixture was extracted five times with ether. The ether layer separated with difficulty, due to the formation of an emulsion with sticky matter. The aqueous layer was retained for further examination.

Treatment of the Ether Layer: Separation of Oxyacanthine and Berbamine

The pale yellow colored ether layer was washed twice with water, and the ether was removed leaving a sticky mass (about 5 Gm.) which was Soxhletted with ether (50 cc.) which dissolved most of it. The solution was filtered, and the filtrate was left for a day or two in a refrigerator during which time colorless crystals, sticky with resinous matter, separated. These were treated with a small quantity of dilute hydrochloric acid (5%), when they went in solution. The solution was filtered and allowed to stand in a refrigerator for a long time, when a very small quantity of the crystals of a hydrochloride appeared. The mother liquor was retained for further examination.

The Crystals.—The pale yellow crude hydrochloride was dissolved in warm water and treated carefully with ammonia, which precipitated an amorphous oxyacanthine base. The base was dissolved in ether, and the ethereal solution on long standing in a refrigerator yielded a minute quantity of colorless needles of oxyacanthine, which when crystallized from methanol melted at 216° *in vacuo*.

The free base was not colored by ferric chloride solution. The hydrochloride of the base, however, was colored green by ferric chloride solution, on gentle warming. The hydrochloride gave a blue coloration with a mixture of ferric chloride and potassium ferricyanide solution. The free base dissolved in concentrated nitric acid with a yellow color and was not colored by concentrated sulfuric acid, but on further mixing with concentrated nitric acid became reddish brown.

The Mother Liquor.—The mother liquor, cooled and treated with sodium nitrate solution, deposited a very small quantity of crystals of berbamine nitrate. The free base was liberated from the aqueous solution on treatment with ammonia, and was recrystallized from methanol, m. p. 155° (dec.). The base gave the same color reactions as the above base, oxyacanthine. Its hydrochloride, which was obtained as colorless needles, recrystallized from water and melted at 264° *in vacuo*.

The melting point and color reactions of the bases indicate them to be oxyacanthine, m. p. 216–217° *in vacuo*, and berbamine, m. p. 156° (dec.), respectively.

Treatment of the Aqueous Layer: Separation of Berberine, Neprotine, and Palmatine

The clear brown solution was treated with hydrochloric acid to give a 0.1% solution of the aqueous extract and allowed to stand overnight in a refrigerator. The yellow hydrochloride of the base, which was subsequently identified to be berberine, gradually crystallized out and was filtered off. The red hydrochloride of a second base, neprotine, obtained first from *M. nepalensis* (8), separated with the yellow hydrochloride. The yellow and the red hydrochlorides weighed 3 Gm. together, and were separated by fractional crystallization from ethanol. The filtrate was retained for further examination.

The Yellow Crystals.—These were purified by repeated crystallization from ethanol, and were identified to be crystals of berberine chloride by the examination of some of the compounds of the base, berberine, liberated from the chloride, for neither the base nor its chloride has a sharp melting point to characterize it.

The Acetone Compound.—Berberine chloride (0.2 Gm.) was dissolved in water (20 cc.) and was mixed with 10% sodium hydroxide solution (4 cc.), heated to 50°, mixed with acetone (10 cc.) and allowed to cool when lemon yellow crystals (0.12 Gm.) gradually separated, m. p. 166–168° (dec.). The crystals did not depress the melting point of an authentic sample of the acetone compound of berberine, m. p. 167–168° (dec.).

Tetrahydroanhydroberberine.—Berberine chloride (4.0 Gm.) was dissolved in water and reduced by zinc dust and acetic acid by heating on the water bath, until the color of the mixture became pale yellow. The mixture was filtered, cooled, and treated with ammonia until the precipitated zinc hydroxide went in solution. It was then extracted repeatedly with ether, and on removal of ether, a colorless crystalline product (3.0 Gm.) was obtained, which was recrystallized from methanol, m. p. 172°. The crystals did not keep well, and they turned yellow gradually. They did not depress the melting point of tetrahydroanhydroberberine, m. p. 173°, prepared from an authentic sample of berberine.

The Red Crystals.—The coppery-red crystals of the hydrochloride were purified by crystallization from ethanol, and were identified to be the hydrochloride of the base, neprotine, by the preparation and comparison of the characteristic picrate.

An ethanolic solution of picric acid (1 mole) was added to a warm ethanolic solution of the hydrochloride (1 mole), from which, upon cooling, shining orange-colored crystalline needles of neprotine picrate were obtained, m. p. 208–210° (dec.). The needles did not depress the melting point of the picrate, m. p. 208–210° (dec.), of neprotine isolated from *M. nepalensis* (8). Found: N, 9.7. $C_{38}H_{38}O_{12}N_4$ requires N, 9.5%.

Examination of the Filtrate.—The filtrate was treated with sodium acetate solution, followed by an aqueous solution of potassium iodide (1:1). The precipitated iodide (3.5 Gm.) was collected and the solution was rejected.

The iodide was dissolved in warm water (1000 cc.) and the solution was reduced by zinc dust and glacial acetic acid until the color of the solution was faintly yellow. The solution was filtered from unreacted zinc, and was treated with ammonia until the precipitated zinc hydroxide redissolved. The solution was extracted repeatedly with ether. The aqueous layer was rejected, and the ether layer was shaken with 3% potassium hydroxide solution (10 cc.).

Treatment of the Ether Solution.—The ether solution was evaporated and the pale yellow residue (2.5 Gm.) was dissolved in methanol. On fractional crystallization, colorless crystalline tetrahydroanhydroberberine, m. p. 172° (*loc. cit.*), was separated from tetrahydroanhydropalmatine which was obtained in small quantity, and melted at 143–144°. Mixed with an authentic sample of tetrahydranhydropalmatine, it melted at 144° showing no depression in its melting point.

The Alkaline Solution.—The solution was acidified and processed in the usual way but insufficient product was obtained to enable identification.

SUMMARY

1. The bark of *Mahonia Griffithii* Takeda which grows in Bhutan, yields altogether five alkaloids, *viz.*, oxyacanthine, berbamine, and palmatine in traces, berberine (0.3 per cent), and neprotine (0.001 per cent).

2. This is the first instance of the occurrence of palmatine in *Mahonia* species.

3. Neprotine was previously obtained from *M. nepalensis* D. C. (8), which occurs in Nepal and to a certain extent in Darjeeling.

4. Neprotine has not yet been obtained from other species of *Berberis* and *Mahonia* growing in different parts of the world.

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Note

A Note on an Air-Activated Pyrex Flowmeter for Biological Experiments*

By FREDERICK F. ANDERSON and ANNE CAMERON

THE flowmeter to be described would appear to have the following advantages for physiological experiments: (*a*) It is made entirely of glass and so avoids the contamination of physiological fluids with traces of metal in experiments in which the fluid is re-perfused. (*b*) It is air-activated, which makes it convenient for the usual laboratory. (*c*) The measurement of flow is continuous, since during the flowmeter's cycle of emptying, the fluid inflow is interrupted; after completion of the emptying cycle, the accumulated fluid at the inflow tube falls rapidly through into the measuring chamber. (*d*). It possesses a wide range of sensitivity and can measure accurately flows of from one drop to over 100 ml. a minute. Higher rates of flow could be accommodated by increasing the dimensions of the apparatus, notably the size of the measuring chamber itself (10 ml. in present model) and the diameter of the inflow tube.

Figure 1 is a sketch of the flowmeter. On *A* is placed a tubing from a low-pressure air supply. A tap off that tube by means of a "T" is connected to a Marey tambour which marks on a kymographic record each emptying of the flowmeter.

For assuring a constant flow of clean air from a line of varying pressures, we have found the Koby Air Purifier and Flow Equalizer[1] very satisfactory.

With the apparatus empty, the air passes up through *B* and out the holes *C* in the top cap of the flowmeter. The cap keeps dirt and dust from contaminating the apparatus with its contained perfusate. The perfusate whose rate of flow is to be measured drops into the opening at *D*, past the glass valve *E*, through the side-arm *F* into the main measuring chamber *G*. As the level of the fluid rises in the measuring chamber *G*, it lifts the glass bulb *H*, until the small rubber disk *I* occludes the opening *B*. The air pressure developing within the chamber *G* forces fluid back through *F* and so raises the valve *E* against the ground glass ring *J*. With all other escape of the fluid shut off, the accumulated air pressure forces the fluid out through the side arm *K*. As air follows the fluid out the side arm *K* the support for *H* is lost, and it drops to the bottom of the measuring chamber, so that air can once again escape through the exit *B*. Simultaneously, the support for valve *E* is lost, so it drops away from *J*, and the accumulated inflow fluid above *J* passes down past *E* through *F* into *G*. *L1* and *L2* represent, respectively, the lower and upper solid glass guides for the float. The cap *M* is attached to the apparatus through a standard taper joint *N* to facilitate the cleaning of the apparatus. The apparatus is both sensitive and accurate and requires no critical adjustments.

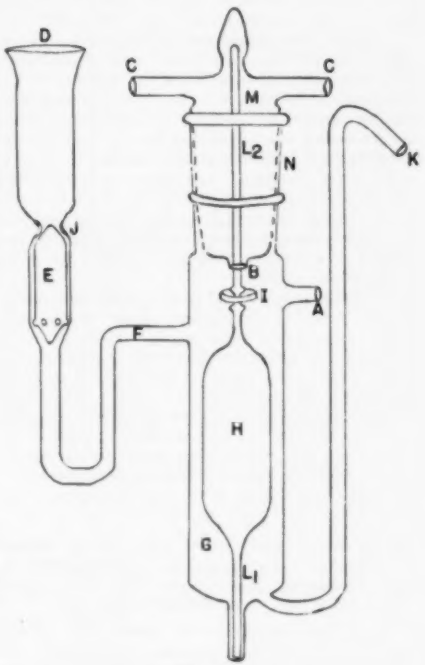

Figure 1

* Received November 15, 1949, from the Research Department, Division of Macrobiology, Ciba Pharmaceutical Products, Inc., Summit, N. J.

[1] Purchasable from Koby Corporation, 139 High Street, Boston 10, Mass.

Book Reviews

Tablet Making. By ARTHUR LITTLE and K. A. MITCHELL. Northern Publishing Co. Ltd., Liverpool, 1949. 121 pp. Illustrated. 14 x 22 cm. Price 15*s*.

This is a long-overdue book which is welcome to those who have sought an introduction to tablet manufacture. It describes the general construction of the various machines, not only tablet compressors, but also coaters, granulators, driers, and mixers. Briefly but clearly the authors direct the adjusting and setting of different types of machines, and discuss the common difficulties that may be encountered in compression, due to excess moisture, improper granulation, improper or insufficient lubricant or binder, etc. Included, also, is a section devoted to small-scale manufacture of tablets, wherein is listed the minimum equipment for satisfactory and efficient production. In the last of the book is a list of formulas, including many of the B. P. tablets, which gives the kinds and amounts of excipients and the procedures which have been found suitable for the particular products.

This book should be of considerable value to industrial libraries, serving as a guide to the newcomer to the tablet department. It is a must for the colleges of pharmacy who teach manufacturing. Also, it will be of value to the hospital or retail pharmacist who contemplates or is now engaged in tablet manufacture.

It is to be regretted that the book is without references and does not lead the reader to original literature covering special tableting problems or to more advanced reading on the general subject.

The Rat in Laboratory Investigation. Ed. 2. Edited by EDMOND J. FARRIS and JOHN Q. GRIFFITH. J. B. Lippincott, Philadelphia, 1949. xvi + 542 pp. Illustrated. 15 x 23.5 cm. Price $15.

The mere appearance of the second edition of this book will be good news to many investigators who have been keenly aware that the supplies of the first edition were exhausted.

Although not severely revised, the new edition has been modernized with critical additions. Pharmacologists who may feel that this field is sadly neglected will be compensated in part by the extensive revisions in the chapter on dosages.

Any investigator in the biological sciences should investigate the scope and contents of this book. Probably never before has such a complete text been written about an animal. A few typical headings of the 22 chapters are: breeding, diet, metabolism, hematology, surgery, and diseases. Following the chapter on gross anatomy are detailed chapters on the principal systems. Each phase of the text is supported by a strong bibliography and illustrations.

While it is true that in a task of this magnitude each specialist might have written his subject somewhat differently, he will think that the others are very well written, and one could ask for very little more.—L. W. HAZLETON.

Manuel théorique et pratique d'Histologie. By GEORGES DUBREUIL and ALBERT BAUDRIMONT. Vigot, Paris, 1950. 635 pp. Illustrated. 21 x 27 cm. Price 3000 francs.

This theoretical and practical guide to the student of histology, by professors of the school of medicine in Bordeaux, is internationally known and renowned. The former editions have been translated into Portuguese.

The fourth edition embodies 627 pages and 730 figures; it provides a wealth of information bearing on pharmacy. It masterfully correlates the problems of structures, both histological and cytological, with the problems of functions as studied from the point of view of the physiologist, endocrinologist, and, last but not least, the pharmacist. For instance, Chapter 15, entitled "Endocrine Glands," discusses the relationship between the nervous system and the various hormones, and Chapter 10, dealing with the teguments (skin, hair, sebaceous glands), is of special interest to those preparing ointments and interested in the penetration of drugs through the skin.

While histology is not of primary concern to the pharmaceutical student, still fundamental notions of histology, as available from this book, are a prerequisite for the understanding of physiology and physiopathology, which are the sciences on which the art of healing is grounded. Emphasis on the chemical aspects of pharmacy tends to overshadow the fact that chemical agents, when used as drugs, are meant to act on an individual, who reacts as a whole, according to the setup defined by histology.—J. DUFRENOY.

The Analytical Chemistry of Industrial Poisons Hazards, and Solvents. Ed. 2. Chemical Analysis series, Vol. I. By MORRIS B. JACOBS. Interscience Publishers, New York, 1949. xviii + 788 pp. Illustrated. 15 x 24 cm. Price $12.

The second edition of this book retains the original organization but has been revised and enlarged to contain the newer information that has become available. There are very thorough chapters on sampling, estimation of dust silica and heavy metals, analysis of the poisonous halogens, hydrocarbons, alcohols, glycols, and ethers, the acids esters, aldehydes, ketones, phenolic compounds, aniline, and miscellaneous organic nitrogen compounds.

This book has a very suitable arrangement by presenting under the heading of each individual compound some description of the uses of the compound, thus indicating where one might expect it to constitute a hazard, then briefly the physiological response to the compound, and finally the detailed procedure for detection and determination of the material. In most cases there is also stated the allowable limits of each of the toxic substances. Thus, one not only finds it useful for laboratory procedures but also a handy reference for the general subject of industrial toxicology.

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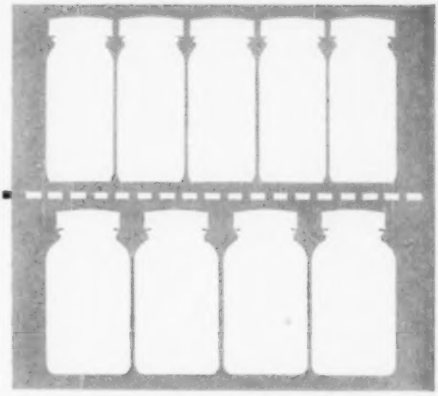

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